

University of Warwick institutional repository: <http://go.warwick.ac.uk/wrap>

A Thesis Submitted for the Degree of PhD at the University of Warwick

<http://go.warwick.ac.uk/wrap/37003>

This thesis is made available online and is protected by original copyright.

Please scroll down to view the document itself.

Please refer to the repository record for this item for information to help you to cite it. Our policy information is available from the repository home page.

ESTABLISHMENT AND ANALYSIS OF BACTERIAL BIOFILM
SUBJECTED TO FLUID FLOW UNDER VARYING NUTRIENT
CONDITIONS.

HILARY SIMMONS B.Sc.(HONS).

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

UNIVERSITY OF WARWICK DEPARTMENT OF BIOLOGICAL SCIENCES.

SEPTEMBER 1988.

TABLE OF CONTENTS

CHAPTER ONE

INTRODUCTION

		1
1.	The aims of the present study.	1
2.	The significance of microbial adhesion.	3
3.	Why do biofilms form at surfaces?	5
4.	How cells attach to surfaces - the mechanisms of attachment.	8
4.1	Observed sequence of biofilm formation.	8
4.2	Physicochemical theories explaining the observed sequence of attachment at surfaces.	10
4.2.1	The DLVO theory.	10
4.2.2	The thermodynamic approach.	13
5.	Effects of enviromental variables on adhesion.	15
5.1	The effect of shear stress on attached biofilms.	17
5.1.1	The effect of substrate concentration on biofilms exposed to flowing conditions.	25
6.	Comparison of activities of freely suspended and attached microorganisms.	28
7.	Uptake of glucose by <u>Pseudomonas fluorescens</u>	29
8.	The approach adopted in this study.	32

CHAPTER TWO

MATERIALS AND METHODS USED IN THIS STUDY

1.	Introduction.	33
2.	Maintenance and growth of stock cultures.	33
3.	Preliminary experiments: the selection of suitable organisms and medium for use in the	

	test rig.	34
3.1	Introduction.	34
3.2	Surfaces considered.	35
3.3	Growth and attachment of cells.	35
3.4	Results.	37
3.5	General conclusions.	42
4.	The flow rig.	42
4.1	Description of the flow rig.	42
4.2	Sterilisation of the flow rig.	48
4.3	Rig cleaning procedures.	49
5.	Procedure used during an experimental run.	49
5.1	Procedure for cleaning the test sections.	49
5.2	Preattachment of a monolayer of cells to the inside surface of the test sections - the 'attachment incubation'.	50
5.3	Preparation of the rig at the start of the run and procedure during the run.	51
5.4	Method of counting the number of cells attached to the tubes.	53
5.5	Calculation of the percentage detachment for various flow rates and run times.	53
6.	The problems encountered with the original experimental procedure.	54
6.1	The effect of reducing the volume of culture inoculated into the tubes and increasing the incubation time.	55
6.2	The effect of changing the cleaning procedure and using a detergent solution during sonication.	59

6.3	Counts of attachment levels using a radiolabelling technique.	68
6.3.1	Prelabelling procedure.	71
6.3.2	Note on the scintillants used.	73
7.	Measurement of the uptake of glucose.	73
8.	Calculation of glucose uptake.	74
9.	Improvement of the attachment incubation procedure.	77
10.	Chemicals.	78

CHAPTER THREE

ANALYSIS AND CHARACTERISTICS OF BIOFILM 80

1.	Detachment studies.	80
1.1	Introduction.	80
1.2	Results and Discussion.	80
1.2.1	Effect of flow rate on detachment.	81
1.2.2	The change in detachment with time.	83
1.2.3	The effect of glucose concentration on detachment.	83
2.	Effect of increasing experimental run length.	86
2.1	Introduction.	86
2.2	Results and Discussion.	86
3.	Number of cells remaining on the tube at the end of an experiment.	92
3.1	DNA staining technique using Hoechst 33258.	93
3.1.1	Improvement of the sonication procedure.	97
3.1.2	Problems with different batches of Hoechst 33258.	99
3.1.3	Variation of the concentration of detergent.	101
3.1.4	Temperature of samples.	101

3.1.5	Experiment to check whether the detergent was affecting fluorescence readings.	104
3.2	Thymidine labelling.	107
3.3	The use of various surfactants to remove cells from surfaces without loss of viability.	114
3.3.1	Preliminary experiments to establish the best method of agitating the tubes during the incubation period.	117
3.3.2	Investigation of a number of different surfactants.	120
3.3.3	The use of the surfactant technique to determine numbers of cells at the end of an experiment using the flow rig.	125
4.	Uptake of glucose by free-living cells.	126
4.1	Glucose uptake rates for <u>Pseudomonads</u> .	128

CHAPTER FOUR

1	<u>THE EFFECT OF SHEAR STRESS</u>	130
1.	Introduction.	131
2.	Results and Discussion.	134
3.	Summary and Interpretation.	137

CHAPTER FIVE

	<u>THE EFFECT OF SUBSTRATE CONCENTRATION</u>	144
1.	Introduction.	144
2.	Results and Discussion.	146
3.	Summary.	153

CHAPTER SIX

	<u>GENERAL CONCLUSIONS</u>	156
--	----------------------------	-----

1.	Detachment studies.	156
2.	Increase in uptake of attached cells with time.	156
3.	Effect of shear stress and substrate concentration.	158
4.	Comparison of uptake by attached cells and freely suspended cells.	159
5.	Enumeration of attached cells.	159
6.	Study of monolayers rather than thick biofilms.	160
7.	Limitations of the biofouling rig.	162
8.	Improvement of the flow rig.	162
9.	Possible future studies.	163

REFERENCES

166

APPENDIX

189

1.	Results of uptake experiments.	189
2.	Data for <u>Pseudomonas fluorescens</u> .	197
3.	Data on fluid flow characteristics in the test sections.	201
3.1	Average velocity.	201
3.2	Reynolds number.	201
3.3	Peclet number.	201
3.4	Calculation of shear stress at the pipe wall.	202
3.5	Velocity profiles.	205
3.5.1	Laminar flows.	205
3.5.2	Turbulent flows.	206

LIST OF FIGURES

FIGURE		page
1.1	Interaction free energy (G) versus separation (h) curves as a function of electrolyte concentration (c) for a charged particle approaching a macroscopic surface of the same sign.	12
1.2	Biofilm progression in turbulent flow.	18
1.3	Pathways for glucose catabolism in <u>Pseudomonas fluorescens</u> .	30
2.1	Change in attachment with length of incubation period for cells grown in glucose medium for <u>Pseudomonas fluorescens</u> cells.	38
2.2	Change in attachment with length of incubation period for cells grown in glucose-gelatin medium for <u>Pseudomonas fluorescens</u> cells.	38
2.3	Change in attachment with length of incubation period for cells grown in glucose-glycerol medium for <u>Pseudomonas fluorescens</u> cells.	39
2.4	Change in attachment with length of incubation period for cells grown in glucose-galactose medium for <u>Pseudomonas fluorescens</u> cells.	39
2.5	Effect of culture age on attachment for <u>Pseudomonas fluorescens</u> cells.	40
2.6	Attachment of <u>Pseudomonas fluorescens</u> cells which were grown up and attached in glucose medium at various pH values.	41
2.7	Effect of temperature during the attachment	

	incubation period on <u>Pseudomonas fluorescens</u>	
	cells which were grown up and attached in	
	glucose medium at various pH values.	41
2.8	Diagram of the flow rig.	44
2.9	Photograph of the flow rig.	45
2.10	Photograph of the flow rig.	46
2.11	Effect of varying the length of the attachment	
	incubation period: up to 1.5 hours.	57
2.12	Effect of varying the length of the attachment	
	incubation period: up to 24 hours.	57
2.13	Effect of cell density on attachment level.	58
2.14	Diagram of the flow system for attaching	
	cells to the stainless steel tubes.	79
3.1	The effect of flow rate on detachment of	
	cells.	82
3.2	The effect of time on detachment of cells.	84
3.3	The effect of glucose concentration on	
	detachment of cells.	85
3.4	Uptake versus time for attached cells exposed	
	to a flow rate of 2 l/min and glucose	
	concentration of 0.2 g/l.	87
3.5	Uptake versus time for attached cells exposed	
	to a flow rate of 2 l/min and glucose	
	concentration of 0.05 g/l.	88
3.6	Uptake versus time for attached cells exposed	
	to a flow rate of 2 l/min and glucose	
	concentration of 0.02 g/l.	89
3.7	Calibration curve for calf thymus DNA	
	supplied by Aldrich.	96
3.8	Calibration curve for calf thymus DNA	

	supplied by Sigma.	100
3.9	Change in fluorescence readings with time.	103
3.10	Numbers of cells eluted.	123
3.11	Uptake versus time for freely suspended cells.	127
4.1	Glucose uptake versus flow rate: results of individual experiments.	137
4.2	Glucose uptake versus flow rate during a 30 min run for a glucose concentration of 0.02 g/l.	138
4.3	Glucose uptake versus flow rate during a 1 hour run for a glucose concentration of 0.02 g/l.	138
4.4	Glucose uptake versus flow rate during a 30 min run for a glucose concentration of 0.05 g/l.	139
4.5	Glucose uptake versus flow rate during a 1 hour run for a glucose concentration of 0.05 g/l.	139
4.6	Glucose uptake versus flow rate during a 30 min run for a glucose concentration of 0.20 g/l.	140
4.7	Glucose uptake versus flow rate during a 1 hour run for a glucose concentration of 0.20 g/l.	140
4.8	Glucose uptake versus flow rate during a 1 hour run for a glucose concentration of 2.00 g/l.	141
4.9	Velocity profiles for a distance of up to 10 μm from the surface at different flow rates.	142

4.10	Thickness of laminar sublayer at different flow rates.	143
5.1	Average glucose uptake versus substrate concentration.	147
5.2	Lineweaver-Burk plot: showing the reciprocal of glucose incorporated per cell versus the reciprocal of glucose concentration.	148
8.1	Growth curves for <u>Pseudomonas fluorescens</u> at different glucose concentrations.	198
8.2	Calibration plot showing absorbance versus cell concentration for <u>Pseudomonas fluorescens</u> .	199
8.3	Plot showing cell weight versus cell numbers.	199
8.4	Glucose uptake by a growing culture.	200
8.5	Flow rate versus average velocity through test sections.	203
8.6	Flow rate versus Reynolds number.	204
8.7	Flow rate versus surface shear stress.	204

LIST OF TABLES

TABLE		page
2.1	Attachment levels after improving tube cleaning method: Results of 22/4/85.	62
2.2	Attachment levels after improving tube cleaning method: Results of 24/4/85.	62
2.3	Attachment levles for tubes cleaned in 1% solutions of Lipsol, Micro and Teepol.	63
2.4	Attachment levels for tubes cleaned using solutions of 1% Lipsol, 2% Lipsol, 2% Tween 80 and 2% RBS 25.	65
2.5	Notes on the detergents used.	66
2.6	The effect of using formic acid in the cleaning procedure.	69
3.1	Rate of glucose uptake at different glucose concentrations.	91
3.2	Effect of varying amount of detergent (Triton X-100) on fluorimeter readings.	105
3.3	Effect of changing detergent, stain and DNA concentrations on the fluorimeter reading.	107
3.4	Uptake of thymidine by a growing culture.	110
3.5	Increase in absorbance of culture during experiment on 12/8/86.	112
3.6	Thymidine concentration in the flasks during experiment on 12/8/86: shown by scintillation counts of 50 µl samples of the culture.	112
3.7	Scintillation counts for filtered cells.	113
3.8	The percentage of cells eluted by a 50 ppm Tween 20 solution: measured using an	

	epifluorescent microscope.	118
3.9	The percentage of cells eluted by a 50 ppm Tween 20 solution: measured by scintillation counting.	118
3.10	The percentage of cells eluted by various surfactant solutions: measured using an epifluorescent microscope.	121
3.11	The percentage of cells eluted by various surfactant solutions: measured by scintillation counting.	121

ACKNOWLEDGEMENTS

I would like to express my thanks to Dr M Fletcher and Dr J Duddridge for their supervision and guidance during the early part of this project, and also to Professor D P Kelly and Dr S Y R Pugh, who provided supervision, guidance and support during the latter stages of the project, and helpful comments on this thesis. I would also like to thank Dr I Gosling for his advice during the project.

In addition, I would like to thank Mr J Laws of Harwell Laboratory who built the flow rigs used during this project, and the technical staff of the Biological Sciences Department, University of Warwick.

I would also like to thank my husband for bearing with me during the preparation of this thesis, and the SERC and Harwell Laboratory for financial support during the course of this research.

DECLARATION.

I declare that this thesis is a report of the research undertaken by myself in the department of Biological Sciences under the supervision of Dr. M. Fletcher, Professor D.P. Kelly, (University of Warwick), Dr. J. Duddridge and Dr. S.Y.R. Pugh. (Harwell Laboratory). It is my own original work and has not been presented before.

SUMMARY

The main aim of the project was to study the effect of flow rate (shear stress), under different nutrient conditions on pre-attached bacterial biofilms. The combined effect of flow rate and nutrient supply had not previously been systematically studied, and the relationship between physiological activity, attachment and detachment, growth at a surface, and flow rate appeared to be very complex.

Special experimental flow rigs were built for this project. Thin biofilms (monolayers of cells) were attached to test sections and these were placed in the flow rig. The biofilms were then subjected to different shear stresses at a known glucose concentration. The numbers of cells were small compared to the total volume of medium in the flow rig, and so the change in the substrate concentration during an experiment was insignificant. Radiotracers could therefore be used to measure substrate assimilation at various flow rates and substrate concentrations.

It was hoped to produce a model which would explain the experimental data which was obtained, and assess how flow rate influences biofilm development and substrate uptake. However, the experimental results suggest that flow rate has little influence on glucose uptake by Pseudomonas fluorescens (the species used for this work), and that glucose uptake at various flow rates depends only upon the glucose concentration present in the medium. It is possible that the explanation for this lies in the complex mechanism of glucose catabolism which is reported for this species.

A considerable part of this project was spent in establishing appropriate experimental techniques in order to make the required measurements for analysis of the biofilms in the flow rig.

CHAPTER ONE

INTRODUCTION

1. The aims of the present study

Microbial adhesion may be crudely defined as the association of a microbial cell with the surface of a substratum, involving an interaction between the two which requires an input of energy to separate them. Microbial biofilms are found upon almost all surfaces exposed in an aqueous environment (Characklis and Cooksey, 1983). The study of such biofilms is an important branch of microbial ecology, because they are ubiquitous wherever solid/water interfaces exist. The majority of organisms in any such environment tend to be present as biofilm components rather than freely suspended. Consequently the establishment of stable communities, as well as the simultaneous survival of organisms competing for similar ranges of nutrients in an open system habitat, is a result of biofilm existence. Attachment to surfaces may affect bacterial survival (Herson et al, 1983). In some experiments, attached cells have shown different growth rates (Heukelekian and Heller, 1940, Fletcher and Marshall, 1982), and patterns of metabolic activity (Paul and Jeffrey, 1986; Ladd et al, 1979; Kjelleberg, 1983; Bright and Fletcher, 1983a and 1983b) from freely suspended cells. There are many important economic reasons for studying the effects of biofilms, and improved control of biofilms will only be obtained by a greater understanding of the fundamental aspects of the phenomenon of microbial adhesion and biofilm growth.

The aim of this work was to study the establishment and analysis of bacterial biofilms subjected to fluid flow under

varying nutrient conditions. A considerable amount of research has been carried out to study the effects of various environmental conditions on cells which are attached to surfaces at very low shear stresses, or in quiescent conditions, but as yet comparatively few studies have evaluated the effect of well characterised flow conditions on a well defined population of attached cells, and almost none have combined this with a study of the effects of varying other environmental parameters such as nutrient concentration.

It has been shown by some workers that the rate of biofilm development and its thickness are dependent upon the flow rate, and thus the shear stress. Up to a critical flow rate, the rate of biofilm development has been shown to increase with increase in flow rate (Trulear and Characklis, 1982; Duddridge et al, 1982). This effect is usually assumed to be due to the increased mass transfer of nutrient at higher flow rates. However, the physiology of bacteria may be modified by proximity to a surface and the microenvironment at the interface (Bright and Fletcher, 1983b), and it is possible that this may influence the physiological activity of the cell and contribute to the observed increase in the rate of biofilm development. It was hoped in this study to make some evaluation of whether flow rate influences (i) the kinetics of physiological processes of bacteria in biofilms, (ii) mass transfer of nutrients to, or waste products away from, bacteria in the biofilm, or (iii) both.

The aim of this chapter is to give a general coverage of the current knowledge regarding bacterial attachment, and of work carried out under conditions of fluid flow.

2. The significance of microbial adhesion

Biofilms serve beneficial purposes in natural environments, fulfilling a central role in the cycling of organic and inorganic substrates (Duddridge et al, 1982). They are responsible for removing organic and inorganic 'contaminants' from natural streams and are used in fixed film bioreactors for wastewater treatment processes, such as trickling filters, rotating biological contactors and fluidised beds (Bryers and Characklis, 1982; Duddridge et al, 1982), biotransformations (Bryers and Characklis, 1982) and the production of secondary metabolites. The use of surface-dwelling microorganisms as biological purification filters, to immobilise and potentially degrade dissolved and particulate materials including pesticides, petroleum derivatives, industrial wastes and heavy metals is an area of growing research (Paerl, 1985).

In other cases, 'fouling' due to microbial biofilms (a term used when biofilms have a deleterious effect) can lead to very undesirable effects. Some of these effects, and examples of the situations in which they occur, include:

(i) Problems due to increased fluid frictional resistance: thin biofilms which develop on wetted surfaces in tubes and pipes dramatically increase fluid frictional resistance, even in conduits of large diameter (Characklis and Cooksey, 1983). This is because the cross sectional area available for flow is reduced, and the surface roughness and drag forces are increased (Picologlou et al, 1980). The effect of roughness is magnified for biofilms which contain filamentous organisms (Picologlou et al, 1980).

The energy required to maintain a specific fluid flow through a pipeline increases as the biofilm thickness increases. This causes problems in industrial heat exchangers, water and waste-water pipelines, cooling towers, and other industrial pipelines. In addition, biofilm formation on ship hulls increases drag forces, thereby raising fuel consumption (Characklis and Cooksey, 1983; Trulear and Characklis, 1982).

(ii) Problems due to heat transfer reduction: biofilms which develop on heat transfer surfaces act as insulators and cause a decrease in conductive heat transfer. They may cause a relatively small increase in convective heat transfer, due to the increase in surface roughness of a surface with an attached biofilm. In 1979, Thackery estimated that heat-exchanger fouling (of which microbial slimes are an important component) alone cost the United Kingdom up to £300-500 million per annum (cited by Duddridge et al, 1982).

(iii) Problems due to mass transfer and chemical transformations. These problems occur in many diverse areas. For instance, corrosion may be accelerated by processes occurring in the lower layers of biofilms, causing deterioration of industrial plant (Bryers and Characklis, 1982). Biofilms in drinking water systems can cause a reduction in water quality, and biofilm in industrial production processes can reduce product quality (Characklis et al, 1982). Biofilm formation on teeth causes dental plaque and caries, and attachment of microbial cells to animal tissue causes disease of the lungs, intestinal tract and urinary tract (Trulear and Characklis, 1982; Characklis and Cooksey, 1983).

The significance of biofilms in these diverse fields highlights the need for an increased understanding of the fundamental mechanisms of microbial adhesion and film growth.

3. Why do biofilms form at surfaces?

The microenvironment at the solid-liquid interface has properties which may be quite unlike those of the bulk liquid phase, as conditions at the interface are determined, to some extent, by the properties of the solid substratum and by special physicochemical factors associated with surface phenomena and thermodynamic equilibria. There are thought to be several possible factors which influence the attachment of a bacterium to a particular surface for any given set of environmental conditions. Some of these are listed below:

(i) In some cases the substratum¹ contains a substrate¹ which can be utilised by attached, or closely associated cells. An example of this is bacterial utilisation of organic detritus (Paerl, 1980).

(ii) It is known that a chemically clean surface exposed in an aqueous environment will very rapidly become covered with an adsorbed layer of dissolved molecules (often referred to as a 'conditioning film') which may thus provide an area in which nutrient is concentrated for bacterial colonization (Characklis and Cooksey, 1983; Fletcher, 1980a). This is not likely to be a long-term effect in the case of low molecular weight organic compounds, as the supply of nutrients at the surface will eventually be depleted. However, it may be a

¹ In this study the word substratum has been used to denote the surface, and substrate has been used to denote nutrients.

long term effect if the surface and substrate are oppositely charged (and thus held together by relatively strong forces), or the substrate is amphipathic, i.e. surface active (Fletcher, 1984).

Macromolecular substrates tend to be irreversibly bound to surfaces, and must be broken down by extracellular or membrane-bound enzymes before being utilised by bacterial cells. Their utilization depends on enzyme activity, which itself may be influenced by the proximity of a solid surface. Zobell (1943) pointed out that cells attached at a surface may be at an advantage, because metabolites and exo-enzymes would diffuse more slowly from the interstices between the cell and the surface.

The intrinsic character of a submerged solid is invariably masked through the adsorption of dissolved inorganic and organic substances, as well as through the adsorption of water itself. With smaller molecules, an adsorption equilibrium is generally established, with both adsorption and desorption taking place. Polymers, however, are less prone to desorb because of the large number of anchoring sites (Fletcher, 1980a).

Baier (1980) showed that, in general, substrata of high surface energy and high polarity (ie strongly charged) adsorb macromolecules strongly, the macromolecules being flattened across the surface, whereas low energy, non-polar surfaces bind macromolecules less strongly, but a thicker layer may develop.

Such 'conditioning' films do not always favour adhesion of cells, and in some cases adhesion is reduced (Marshall, 1985).

(iii) The cell surface hydrophobicity of bacteria can vary considerably. Marshall and Cruikshank (1973) suggest that hydrophobic bacteria may be considered as being rejected from the aqueous phase and attracted to any non-aqueous phase, including solid surfaces. Hydrophobicity may thus contribute to the attachment process and affect the orientation of some bacteria at interfaces.

(iv) In many cases the transport of nutrient/oxygen from the bulk phase to attached cells can be rate-limiting. However, in situations where the biofilm is subjected to shear stress, transport of nutrients and oxygen to the cells and removal of waste products, is likely to be more readily facilitated. In these situations the attached cells may be at an advantage (Trulear and Characklis, 1982; Fletcher and Marshall, 1982).

(v) The local microenvironment at the surface could offer protection against unfavourable conditions in the bulk fluid: for example, against lethal agents such as biocides and toxic metals (Herson et al, 1987), phages (Fletcher and Marshall, 1982), and against perturbations and high flow rates in the bulk phase (Atkinson and Fowler, 1974). The surface may provide a microenvironment in which the pH is more favourable (Stotsky, 1966, cited by Fletcher and Marshall 1982).

(vi) It is possible that substrate uptake mechanisms or respiratory efficiency may be affected by surface-induced modifications in cell membrane structure, to the advantage of the attached cells.

(vii) The environment in a biofilm may be advantageous because it allows interactions between cells in the film, or facilitates extracellular enzyme activity. Interactions between autotrophs such as diatoms and heterotrophs such as

bacteria, may occur as a result of excretion of photosynthetically produced soluble organic carbon. There are, however, many experimental problems to be solved before this can be proved (Characklis and Cooksey, 1983).

4. How cells attach to surfaces: the mechanisms of attachment

4.1 Observed sequence of biofilm formation

Cells are transported to surfaces by a variety of forces, including sedimentation, chemotaxis, Brownian motion, fluid dynamic forces and the effects of cell surface hydrophobicity (Marshall 1986).

Marshall et al (1971) observed that the sorption of two marine bacteria to glass surfaces involved an instantaneous reversible phase, and a time-dependent irreversible phase. He found the following differences between the two phases of attachment:

(i) Reversible attachment: in this phase the cells exhibited Brownian motion, they could be removed by washing the surface with 2.5% NaCl, and in some cases rotated around a fixed pole.

(ii) Irreversible attachment: in this phase the cells no longer exhibited Brownian motion, were not removed by washing with 2.5% NaCl, and did not rotate.

A recent study (Lawrence et al, 1987) suggested that surface colonization by Pseudomonas fluorescens can be subdivided into the following sequential colonization phases:

(i) a motile attachment phase, during which cells were freely motile while attaching; (ii) a reversible attachment phase, in which cells attached apically, maintained a fixed position on the surface, and rotated; (iii) an irreversible attachment phase, in which cells became attached longitudinally rather

than apically; (iv) a growth phase, in which attached cells increased in length, then divided, separated, moved apart laterally and slid next to one another, permitting tight cell packing and simultaneous growth and adherence, and (v) a recolonization phase, occurring after approximately four generations, in which individual cells emigrated from developing microcolonies to colonize the surface at new locations.

ZoBell (1943) suggested that firm attachment of an attached bacterium at a surface required several hours: he postulated that this was due to the need for synthesis of extracellular polymer adhesives. In 1970, Corpe showed that copious amounts of 'slimy' extracellular polymers can in fact be produced in microbial films. Polymer footprints remaining on surfaces after the removal of attached cells were found by Marshall et al (1971). Subsequently, polymer bridging was shown directly by using solid substrata which could be sectioned at the adhesion surface (Marshall and Cruikshank, 1973; Fletcher and Floodgate, 1973). Many studies have now shown polymer bridging occurs in all cases in which bacteria are irreversibly bound (Costerton et al, 1981).

As yet, little information concerning the structural analysis of purified extracellular polymer adhesives in microbial systems is available. Polysaccharides have long been held responsible for the bridging between cells and substrata during the irreversible phase of adhesion, although their role in the initial events has been unclear (Fletcher and Floodgate, 1973). It has been shown, however, that the production of extracellular polysaccharides is not directly related to cell adhesion, and that they may actually inhibit adhesion (Pringle et al, 1983). The results of various

qualitative studies which were summarised by Characklis and Cooksey (1983), indicated that such polymers are likely to be glycoproteins.

4.2 Physicochemical theories explaining observed sequence of attachment at surfaces

Various physicochemical theories have been put forward to attempt to explain, with some degree of success, the mechanisms which influence bacterial adhesion at surfaces. A brief summary of the two main theories is given here.

4.2.1 The DLVO theory

The Derjaguin-Landau and Verwey-Overbeek (DLVO) theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948), was developed to consider the electrostatic charges on colloidal particles. This theory suggests that the total interaction between two particles comprises two additive terms: one due to van der Waals forces (G_A), and one due to the overlap of the electrical double layers (G_E), associated with charge groups present on the particle and macroscopic surfaces (Rutter and Vincent, 1980). This theory can be adapted to consider the charges on bacterial and substratum surfaces, and has been used to explain the two stage attachment process observed in the study of Marshall et al (1971).

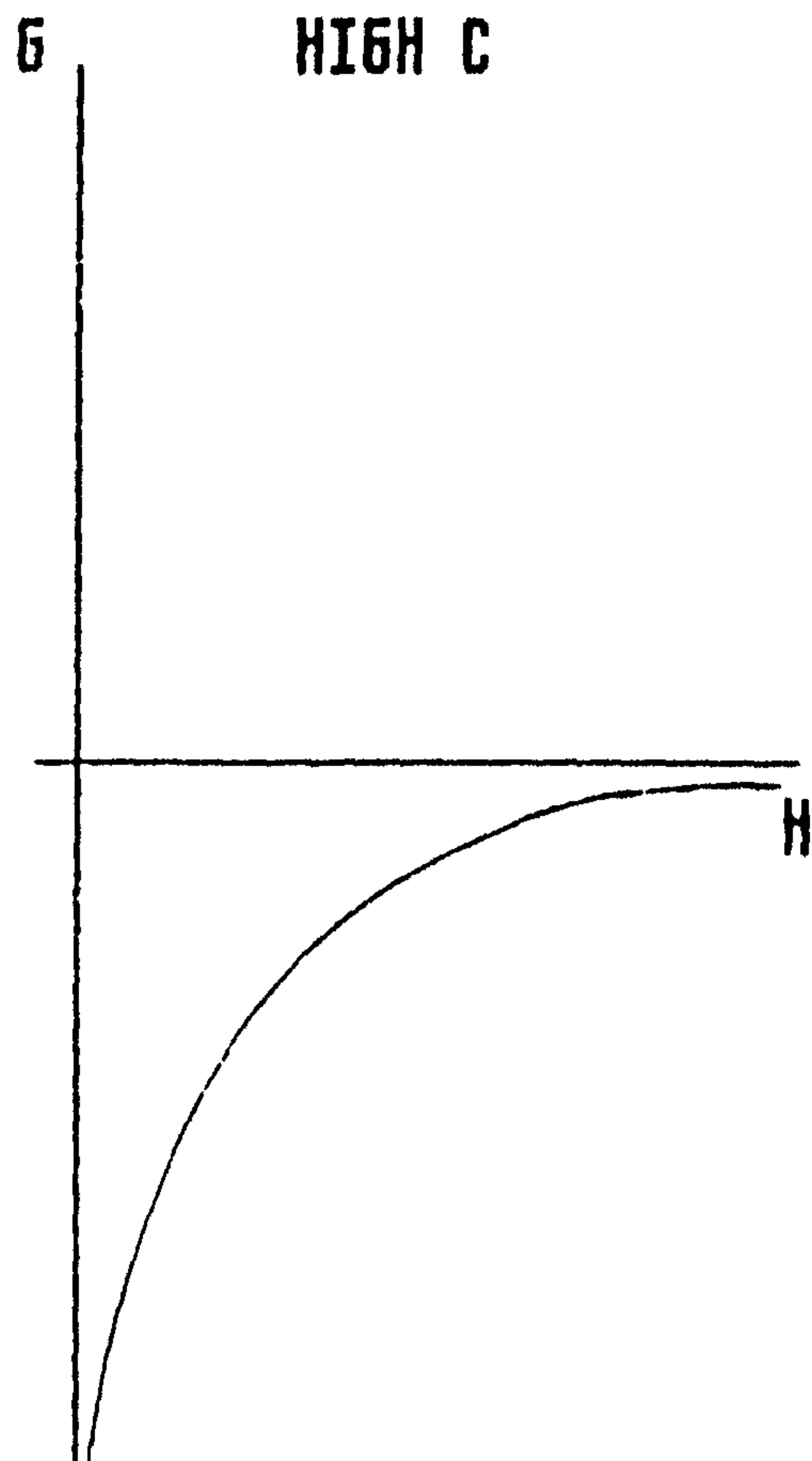
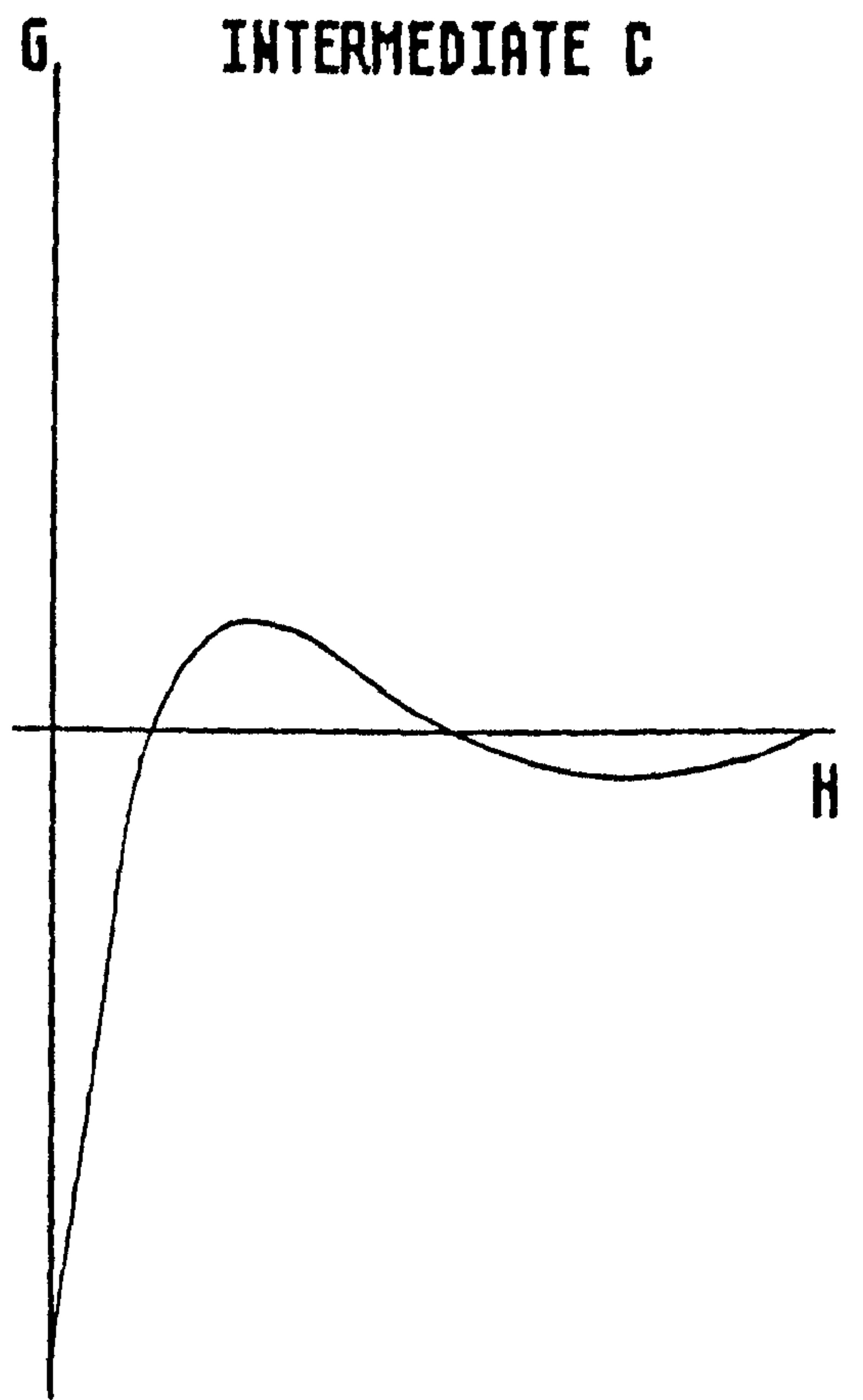
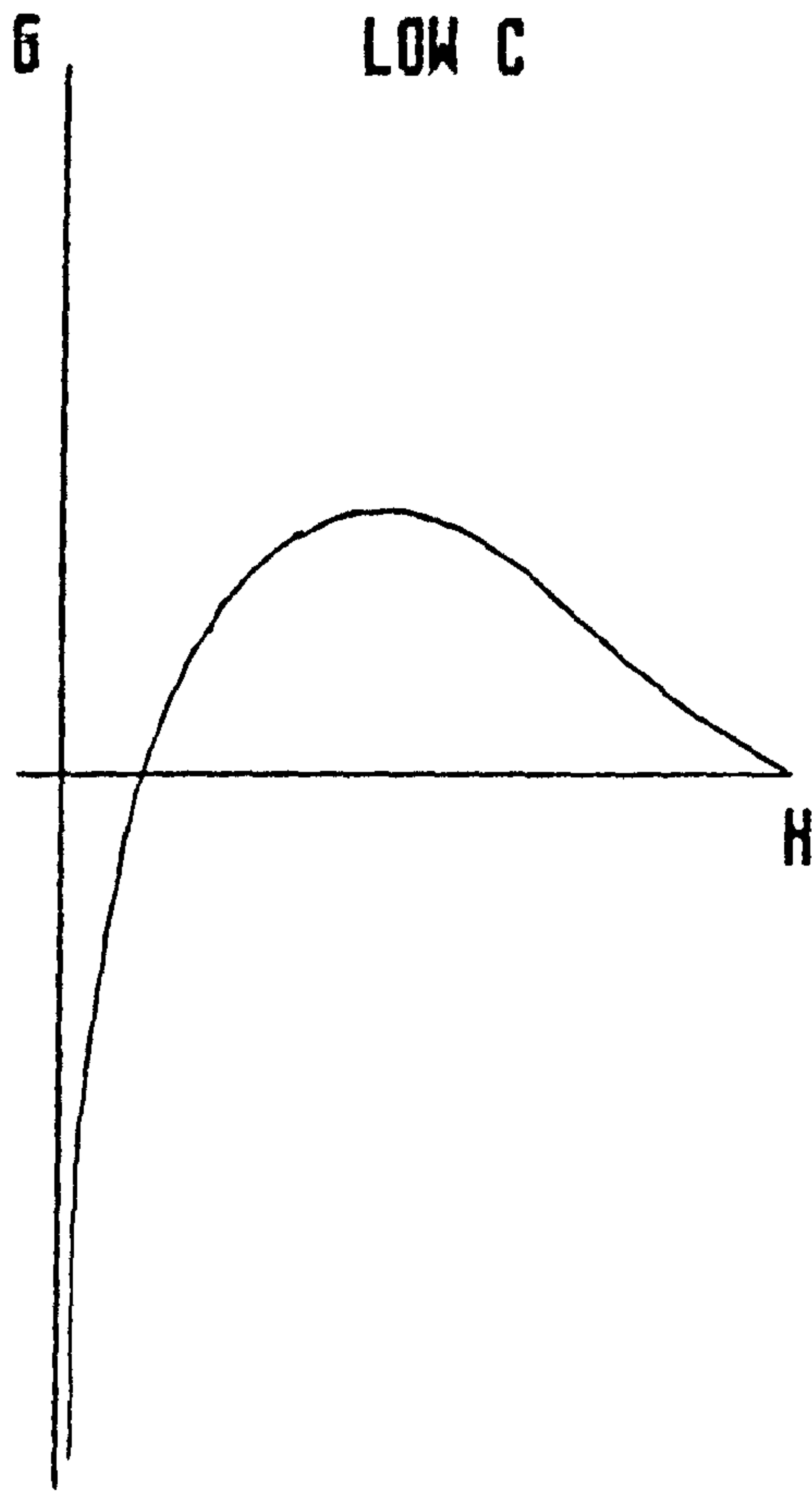
Most solid surfaces found in natural habitats possess a net negative charge (Marshall, 1985), and in most conditions bacterial cells are also negatively charged. Negatively charged surfaces in aquatic environments are surrounded by a diffuse layer of positively charged ions (Fletcher, 1979b). When a bacterium approaches a surface, the degree of attraction or repulsion depends on the thickness of this double

layer, which in turn depends on the valency and concentration of the electrolyte. The DLVO theory predicts a strong attraction between surfaces at a very close range (the 'primary minimum'), an electrostatic barrier at a longer range and then a weaker attraction region (the 'secondary potential minimum') at a slightly greater separation of surfaces (see Fig. 1.1). When bacteria are held by forces at a particle separation corresponding to the secondary minimum, they are not actually in contact with the surface, and this is thought to be the position of bacteria which are reversibly attached (Marshall et al, 1971). The bacterium itself cannot overcome the repulsion barrier which is present closer to the surface.

Belas and Colwell (1982) suggested that in the case of species of Vibrio, in which there is a dual occurrence of sheathed, polar flagella and lateral flagella, initial reversible adhesion results from contact of the sheathed polar flagellum with the surface. After about 3 hours the production of lateral flagella begins; at this stage it is still possible for the cell to move on the substratum. The lateral flagella would be intermediate structures between reversible adhesion and subsequent irreversible adhesion involving polymer bridging.

Tadros (1980) calculated that extracellular polymers with a radius of less than 2 nm can make contact with the surface under Brownian motion. Once these filaments are deposited in the primary minimum they become firmly bound as a result of the high attractive energies at this point.

1.1 Interaction free energy (G) versus separation
(h) curves as a function of electrolyte
concentration (c) for a charged particle
approaching a macroscopic surface of the same
sign.



4.2.2 The thermodynamic approach

Many studies have attempted to relate adhesion to surface energy (or related parameters such as hydrophobicity) of the substratum.

Surface free energy (γ_s), can be approximated by determining the critical surface tension. This can be found by calculating the sum of polar and dispersion components of surface free energy, or by determining γ_s from a single contact angle measurement using the equation-of-state approach (Marshall, 1985). Hydrophobicity has been measured in some studies: this is expressed as water contact angle, θ_w (Fletcher and Loeb, 1979), or as the work of adhesion between water and the surface, W_A , (Pringle and Fletcher, 1983).

Baier (1980) called the range of hydrophobic substrata with a surface tension in the range 20 to 30 mN/m "minimally bioadhesive". Various studies have shown minimal adhesion by bacteria on such hydrophobic surfaces, and increased attachment on hydrophilic surfaces (Dexter et al, 1975, Absolom et al, 1983). However, other studies have provided conflicting results (Fletcher and Loeb, 1979). In the majority of cases bacterial cells have shown increased attachment to hydrophobic surfaces (Fletcher and Marshall, 1980). No clear-cut relationship has been found between substratum wettability and levels of attachment.

In some of the studies described above, attachment was related only to the surface free energy of the substratum: the bacterial surface free energy and the liquid surface tension were not taken into account.

If interfacial tensions between all three components in the system, i.e. the cell surface, the solid substratum and

the liquid phase, are taken into account, bacterial adhesion is favoured if the process causes free energy to decrease. The adhesion process is described thermodynamically by the equation:

$$\Delta F^{adh} = \gamma_{BS} - \gamma_{LS} - \gamma_{BL}$$

where ΔF^{adh} is the change in free energy of adhesion and γ_{BS} , γ_{LS} and γ_{BL} are the bacterium/solid, liquid/solid and bacterium/liquid interfacial energies respectively (Fletcher and Marshall, 1982). Adhesion is favoured by a reduction in free energy, i.e. a negative value for ΔF^{adh} . The interfacial energies in each case are determined by the surface free energies of the two phases. In general the more disparate the two surface free energies, the greater the interfacial energies. This applies if electrical charges and specific ligand receptor interaction can be neglected. Various groups of workers have found that observed levels of adhesion can be predicted by considering surface energy (Absolom et al. 1983; Fletcher and Pringle, 1985; van Pelt et al. 1985). There are, however, some studies which show little or no accountable relationship between attachment and surface energies (McEldowney and Fletcher, 1986b).

The success of many experiments has shown that physico-chemical forces can predominate in bacterial adhesive interactions. However the physicochemical models do not predict attachment in every case. Potential problems in the application of the DLVO theory approach were discussed in some detail by Pethica (1980) and Tadros (1980). Pethica (1980) also pointed out some of the limitations of the surface energy approach, and emphasised the connections between the colloid stability (DLVO) and surface energy approaches. As a

simplification, the failure of these models to predict attachment in every case is mainly due to:

(i) the fact that bacteria are assumed to be homogeneous particles, and the chemical and structural complexity of the bacterial surface is not taken into account: the structural chemistry and conformation at the cell surface can vary enormously from one bacterial species and strain to the next (Pringle et al, 1983) and also depends upon the conditions in which a bacterium is grown;

(ii) the fact that bacteria are living dynamic organisms whose physiology can cause their adhesive properties to change over time. For instance, Busscher et al (1986) demonstrated that the time at which a measurement is taken may influence the results of experimental measurements of adhesion.

(iii) the fact that the effect of conditioning films and bacterial competition for attachment sites are not taken into account (Marshall, 1985).

5. Effects of environmental variables on adhesion

The evidence overwhelmingly suggests that bacterial adhesion is primarily the result of physico-chemical interactions between the bacterium, the solid substratum and the liquid phase (see section 4, pages 10-15), and further that adhesion is influenced by changes in environmental conditions. The range of variables that can influence these physico-chemical interactions is immense, and it is possible that quite small changes in any one variable might cause large differences in bacterial attachment levels and/or rates of attachment (McEldowney, 1984).

The bacterial genome determines such features as the cell wall composition, polymer production and the possibility of an active response to an association with a substratum. Cell surface characteristics are influenced by environmental conditions such as the carbon source, the C/N ratio of available nutrients, growth rate, physiological age of the cells, and bulk phase pH and temperature. (The variability in the nature of cell surfaces has a great effect on adhesion, the extent of which depends on the bacterial species.) In addition, the effect of ions binding to the cell surface, and the effect of conditioning films at both the cell surface and the solid substratum, may markedly promote or inhibit adhesion. The final determinant of bacterial attachment to a solid surface will be the balance of physico-chemical forces for and against adhesion, which will be affected by all the above factors.

Many studies on adhesion have considered the effect of varying parameters such as bacterial species, culture age, cell concentration, temperature and pH, substratum type (including the effect of various conditioning layers), substrate and concentration.

Fletcher (1977), showed that attachment of a marine Pseudomonas sp. to polystyrene increased with suspended cell concentration: the attachment curve levelled off as the surface became 'covered with bacteria'. Similarly, the number of attached bacteria increased with the time allowed for attachment, and then levelled out. In this experiment it was also found that attachment declined with increase in culture age. The progressive decrease in cell motility after the onset of the stationary phase was suggested to be the cause

of this. Stanley (1983) also found cell motility was an important physiological variable in attachment: adherence decreased by at least 90% when flagella were removed by blending.

In many experiments, attachment has been shown to increase with temperature, up to a certain level. The effects of changes in pH, substratum type and substrate are much more complex, as was indicated in section 4.2 (pages 10-15).

Studies of the environmental factors which affect attachment have frequently sought to correlate results with theoretical models of adhesion or to study the differences in cell metabolism, etc, between attached and free living cells (see Section 6 in this chapter). Such data gives more information on the ecology of biofilms, and methods of controlling biofilms. The vast majority of these experiments have been carried out either under static conditions, or else the effect of shear stress on the cells has not been well characterised or correlated with the results.

It is hardly surprising, in view of the complexity of the factors affecting adhesion, that many of the results obtained in various different experimental systems would appear to be conflicting. This reflects the present incomplete understanding of the subject.

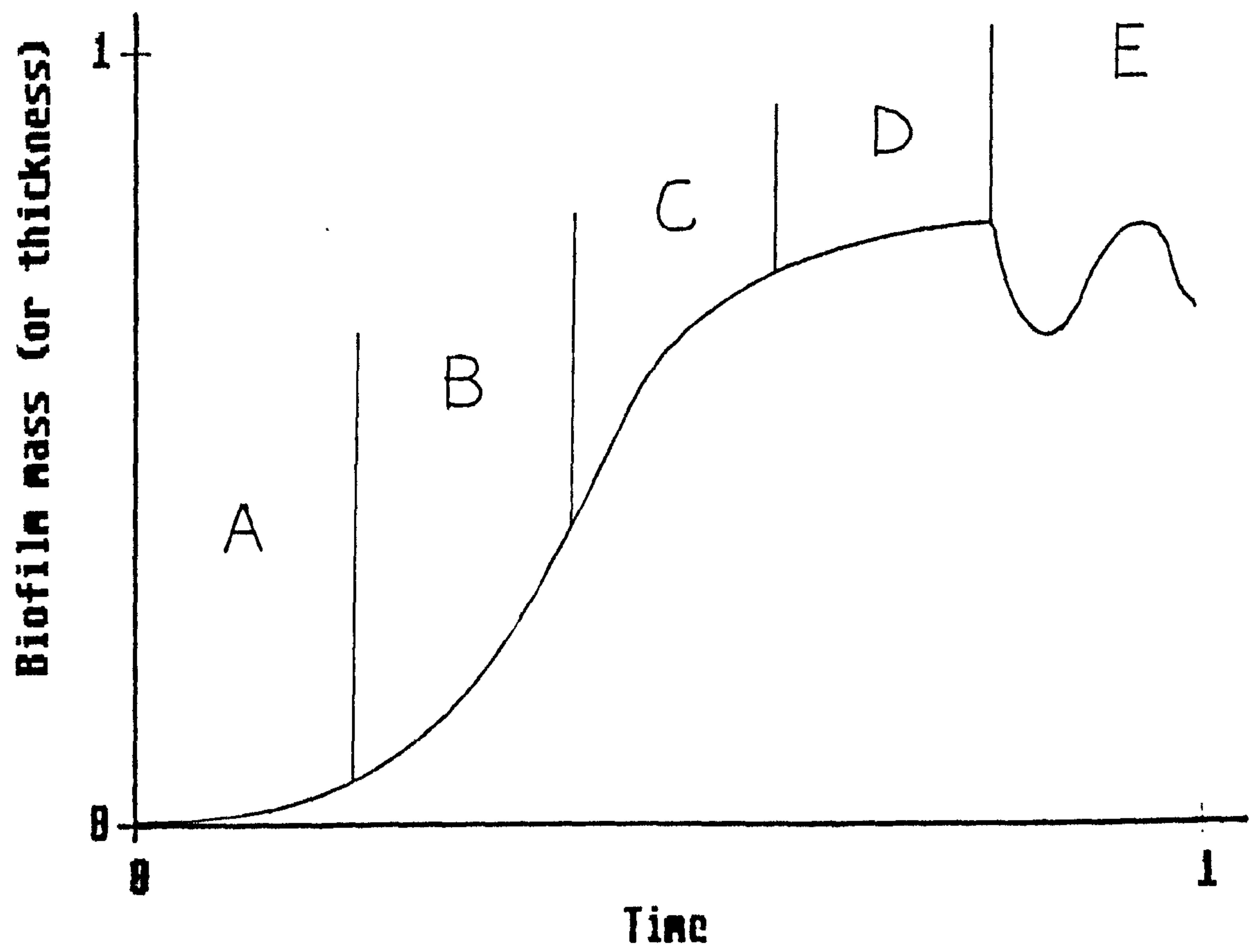
5.1 The effect of shear stress on attached biofilms

Microorganisms growing within surface microenvironments are surrounded by a hydrodynamic boundary layer or dead volume through which nutrients and wastes must diffuse. The size of this boundary layer decreases with increasing flow. Within the boundary layer, inertial forces become relatively unimportant relative to viscous forces (Douglas et al, 1979).

1.2 Biofilm progression in turbulent flow.

Key to symbols:

- A Lag period.
- B Exponential growth.
- C Decreasing rate.
- D Plateau.
- E Sloughing.



Consequently, surface microenvironments are frequently diffusion-limited and attached bacteria may deplete the micro-environment of substrate (Caldwell and Lawrence, 1986).

It has been shown that biofilms develop in a sigmoidal fashion (Bryers and Characklis, 1982) (see Fig 1.2). Evidence suggests that biofilm development is the net result of several transport and reaction rate processes:

(i) Adsorption of dissolved organic molecules at the surface. The formation of these 'conditioning' films has already been discussed.

(ii) Transport of microbial particles to the surface. In a turbulent flow regime convective forces will dominate the transport processes. Eddy diffusion may direct the bacterial cell into the relatively stationary boundary layer near the surface, where it is slowed by frictional drag forces, and by a force exerted by the draining fluid film between itself and the fluid surface. Turbulent eddies and downsweeps of flow may break into the boundary layer, bringing cells close to the surface (Bryers and Characklis (1982).

(iii) Having been brought into contact with the surface, the cells may adhere. As stated earlier, it is generally accepted that adhesion of a bacterial cell to a surface is a two-stage process: reversible adhesion followed by irreversible adhesion. As yet, there is no evidence for the occurrence of the reversible adhesion stage in conditions of turbulent flow; in studies of attachment carried out at very low fluid shear rates, or in quiescent conditions, biofilm accumulation rates may have been mass-transfer limited and, therefore the data are not necessarily applicable to turbulent flow conditions.

(iv) Biofilm production. This is the net accumulation of attached material due to cellular reproduction and microbial production of extracellular polymers.

(v) Biofilm detachment. Portions of the biofilm are continuously removed. The rate and extent of removal is highly dependent on the hydrodynamic conditions. In the case of thick biofilms, 'sloughing' may occur. This is a random large-scale removal of biofilm, attributed to limitations of nutrient/oxygen in the deeper portions of the film.

In a variety of experiments, the effect of shear stress on attached biofilms has been observed. In some cases, the aim of these experiments has been primarily to study the effects of applied shear stress on the biofilm; in other cases the effect of shear stress has been incidental to the experiment, and only one set of hydrodynamic conditions may have been considered (Lawrence et al, 1987; Eighmy and Bishop, 1985). Very few studies in which hydrodynamic conditions have been well characterised have been reported.

Most of the experiments which have been carried out fall into three broad categories. These include:

(i) Experiments in which the primary aim was to define a critical shear stress at which all, or a large percentage of attached cells are detached (Duddridge et al, 1982; Doroszewski et al, 1979; Powell and Slater, 1982), in an attempt to define the 'strength of adhesion'. In many cases it was found that cells detached leaving a residue on the surface, suggesting that the strength of adhesion of the cell to the binding material was actually being determined.

(ii) Experiments in which biofilm formation at a surface has been observed; parameters such as numbers of cells attach-

ing/detaching, growth rates, etc, have frequently been measured. In many cases the aim of these experiments has been to fit the observed data to a model (Powell and Slater, 1983; LaMotta, 1976; Bryers and Characklis, 1981, 1982), or to assess the conditions which will minimise biofilm formation (Pedersen, 1982b), or both.

In some cases the initial formation and build up of the biofilm was investigated (Powell and Slater, 1983; Caldwell and Lawrence, 1986), but usually mature biofilms have been studied (LaMotta, 1976; Rittmann and McCarty, 1980b).

(iii) Other types of study: many studies have taken into consideration other factors beside flow rate, such as pH (Crouch et al, 1985), temperature (Pedersen, 1982b), substratum type (Crouch et al, 1985) substrate concentration (see below), the effect of bacterial species (Powell and Slater, 1983; Crouch et al, 1985), biomass concentration (Bryers and Characklis, 1982), suspended organism growth rate (Bryers and Characklis, 1981), and the effect of the shear stress on the physiology of the bacterial cells (Eighmy and Bishop, 1985). Powell and Slater (1982) attached B cereus cells to glass capillaries, and investigated the effect of increasing the length of the attachment incubation period ('settling time'). The cells were subsequently exposed to increasing shear stresses, and it was shown that the longer the attachment incubation period, the greater the shear stress required to remove all cells from the surface. However, for attachment incubations of more than two hours, the shear stress required to remove the cells remained constant.

Various types of 'bioreactor' have been used in order to

study the effects of shear stress on attached cells. In many of these experiments the hydrodynamic conditions were poorly defined, and in some cases where a correlation with increase in turbulence was attempted, the actual conditions at the surface of the attached cells were not characterised.

Conditions in reactors operating in continuous recycle mode may approximate closely the conditions in some industrial applications, but precise determination of the processes occurring in the reactor is difficult. In other types of reactor, a continuous feed of fresh medium is used. This has the advantage that the concentration of substrate is set by the irrigation solution, metabolic waste products are completely and continuously removed, and the concentration of substrate and/or test compounds can be changed instantaneously. However, such reactors are less practical at high flow rates, and limit use of radiolabels, etc.

Reactors in which biofilms are attached to the inside walls of tubes have been used by Bryers and Characklis (1981, 1982). In some cases the effect of laminar flow through capillary tubing or very closely spaced slides has been studied (Doroszewski et al, 1978; Powell and Slater, 1982 and 1983; Kjelleberg et al, 1982; Caldwell and Lawrence, 1986; Lawrence et al, 1987); microbial growth was quantified in situ microscopically in these experiments. All the advantages and disadvantages of using a 'once through system', described above, apply. The hydrodynamic conditions in this type of apparatus are, however, well characterised. The disadvantages include the fact that only laminar flow rates can be achieved, and the area of biofilm actually observed is small. Pedersen (1982a and 1982b) used a similar system: he inves-

tigated the effect of laminar flows past biofilms attached to banks of microscope cover slips, which were arranged in lamellar piles using special spacers. An individual slide could be removed quickly and replaced by a clean one.

The radial-flow growth chamber (RFGC) has also been used in several biofilm studies (Fowler and McKay, 1980; Duddridge et al, 1982; Crouch et al, 1985). It is made up of two discs at a given distance apart, one of which is fitted with a central inlet pipe. Fluid is pumped in through the inlet pipe, and flows radially outwards between the discs. Thin biofilms are usually studied. The advantage of this equipment is that the effect of a continuous range of flow rates can be studied in one experiment: however because of this, the equipment is unsuitable for uptake studies, such as those carried out in this project.

Another reactor configuration which has been used is the annular reactor (La Motta, 1976; Trulear and Characklis, 1982), consisting of two cylinders, one of which rotates on its axis continuously. These have generally been operated with thick biofilms attached to the cylinder walls, and the flow conditions occurring at the biofilm surface are not well defined.

Other equipment has been used in which the fluid dynamics of the system were very poorly defined. For instance, Nelson et al (1985) used increases in stirring rate as an indicator of the degree of turbulence existing in 'continuous flow bacterial adsorption reactors' (these were Berzelius beakers which contained biofilms attached to glass slides), and Rittmann et al (1986) studied biofilms attached to glass beads in a column reactor.

The experimental rigs used in this study were designed to have several advantages over some of the other types of equipment used for biofilm studies. The special features were:

(i) The flow rigs were of a relatively small size, so that they were easy to sterilise for each experiment; this is much more difficult for larger apparatus.

(ii) The number of cells in the system was small compared to the total volume of medium in the rig, and the experiments were quite short, so the glucose concentration of the medium could be regarded as constant. Thus the rig had most of the advantages of both the recycle and 'once through' modes of operation, without the disadvantages.

(iii) As the length of each experimental run was short, the biofilm was unlikely to be affected by a build-up of metabolic waste products in the medium. The medium in the rig was recirculated, and so the use of radiotracers to study substrate uptake at high flow rates was not prohibitively expensive.

(iv) The thickness of the biofilm would be negligible compared to the pipe diameter, which would mean that the fluid hydrodynamics would be well defined at the surface of the film surface, as fluid dynamics of flow through pipelines is well documented. In addition, the fact that only monolayers were to be studied would mean that the complications which arise because of the existence of different zones in thick biofilms would not occur.

5.1.1 Effect of substrate concentration on biofilms exposed to flowing conditions

In many cases, in quiescent and flowing conditions, it has been noted that attachment/detachment and growth at surfaces is highly dependent upon the nature and concentration of the substrate supplied to the cells. Flowing conditions generally enhance the supply of nutrient to the cell, reducing the likelihood of substrate depletion occurring.

Although the transport of glucose from the bulk liquid to the fluid-biofilm interface may be represented by many expressions, the simplest way is to use that of the concentration driving force, as shown below,

$$N = k_L(s-s_s)$$

where N is the glucose flux to the surface ($ML^{-2}t^{-1}$), k_L is the mass transfer coefficient (Lt^{-1}), s is the glucose concentration in the bulk solution (ML^{-3}) and s_s is the glucose concentration at the film surface (ML^{-3}). Under steady state conditions, the amount of glucose transported to the biofilm surface must equal the amount of glucose consumed by the biofilm (Trulear and Characklis, 1982). LaMotta (1976) investigated liquid phase diffusion and identified two limiting regimes, depending on the magnitude of the mass transfer coefficient. If k_L is large the microorganisms in the biofilm receive a maximum amount of glucose, and the rate of the overall process is determined by the kinetics of the internal diffusion-reaction process. Under these conditions, the kinetics of the internal diffusion-reaction process may be measured, because diffusional resistance resulting from the

liquid phase is insignificant. When k_L is small the substrate concentration at the biofilm surface is essentially zero. The liquid phase diffusional resistance is significant, and the rate of the overall process is determined by the rate of glucose transport to the biofilm surface.

The transition from high to low liquid phase diffusional resistance may be achieved by increasing fluid velocity at the biofilm surface (LaMotta, 1976). Unless liquid phase diffusional resistances are minimized, the substrate removal efficiency of biological film systems may be limited by the transport of reactants from the bulk liquid to the fluid-biofilm interface. Several studies have borne these observations out.

Caldwell and Lawrence (1986) showed that the surface microenvironment becomes substrate-depleted in the absence of sufficient laminar flow velocities and that glucose rather than oxygen was the rate-limiting substrate in their flow cell system. The Monod equation is often used to predict the growth rate of microorganisms. This relates the specific growth rate, μ , to the half-saturation constant, k_M , the maximum specific growth rate, μ_{MAX} , and the substrate concentration, S , as follows,

$$\mu = \frac{\mu_{MAX} \times S}{k_M + S}$$

However, Lawrence and Caldwell's study showed that in the case of microorganisms exposed to flow at surface micro-environments, growth rate is also dependent upon laminar flow velocity. These workers also showed that under conditions of laminar flow and nutrient excess (1 g glucose/l), the growth

rate of Pseudomonas fluorescens was 'independent of flow-rate'. In quiescent conditions, at a glucose concentration of 0.1 g/l, growth rate declined to zero after some hours. When a fluid flow past the surface was initiated, the growth rate resumed at the initial level. Computer-enhanced microscopy was used to measure the numbers of attached cells. However, a limitation of this study was that the effect of a range of different flow rates was not considered. Only one flow rate was actually used and this was compared with uptake under quiescent conditions.

Other workers (LaMotta, 1976; Trulear and Characklis, 1982) have shown that the rate of substrate uptake can be increased by increasing the fluid velocity, until the process becomes reaction-controlled: in this regime the rate is no longer affected by the fluid velocity. Trulear and Characklis (1982) measured biofilm thickness as a function of substrate loading, and found that the removal rate of glucose increased in proportion to biofilm thickness up to a critical thickness, corresponding to the depth of glucose penetration into the biofilm, beyond which removal rate remains constant. The depth of glucose penetration increased with increasing reactor glucose concentration.

Pedersen (1982b) showed an exponential relationship between biofilm production rate and flow velocity within the range of 0 to 15 cm/s. In this case, the biofilm was attached in a system using slowly flowing seawater, in which conditions were laminar, and nutrient levels were below those required to saturate the cells.

It has also been shown that an increase in nutrient loading affects biofilm density and morphology. For instance,

Trulear and Characklis (1982) showed that biofilm density increases with glucose loading rate, and that density and morphology were related. Low density biofilms exhibit a filamentous structure, whereas high density biofilms showed a nonfilamentous structure characterised by dense patches of microbial colonies.

6. Comparison of activities of freely suspended and attached microorganisms

The environmental conditions at a solid-liquid interface differ from those in the bulk aqueous phase, and, accordingly, the physiological activity of bacteria attached to surfaces may differ from that of freely suspended cells.

Many workers have attempted to evaluate differences in attached and suspended cell activity by a variety of methods, including measuring changes in growth of attached organisms (Jeffrey and Paul, 1986a; Kirchman, 1983), respiration (Jeffrey and Paul, 1986b; Bright and Fletcher, 1983b), and substrate uptake/transformation (Simon, 1985; Ladd et al, 1979; Fletcher, 1987). A number of studies have suggested that, in general, attached organisms show increased activity (Jeffrey and Paul, 1986; Fletcher, 1986). Egan (1987) summarised the results of several studies (in which various types of 'activity' measurements had been made), and concluded that for oligotrophic systems in particular, attachment to particles usually increased the activity of microbial populations: however, under some environmental conditions, no stimulation, or inhibition of microbial activity has been observed.

It has been found that the result obtained depends not only on the type of activity being measured, but also upon a

range of experimental conditions: the organism used, the chemical composition of the substratum, the effects of conditioning films, and factors such as the substrate type and concentration. Cells of a particular species may become firmly attached to a particular surface, or only superficially associated with it.

It is not known whether the greater activity which is often associated with surfaces is due to stimulation of activity on attachment, or whether attaching cells are a subpopulation (of a heterogeneous bacterial suspension) which is inherently more active (Paul and Jeffrey, 1986).

There are thought to be three principal ways in which the environmental conditions at a solid surface may influence the physiology of attached cells: nutrient concentration or accessibility may be different at the interface, processes sited in the cell membrane may be modified by elastic deformation of the cell envelope, and also surfaces provide a site for colonisation and the development of a bacterial biofilm, in which cells are embedded in a polymeric matrix, allowing for interactions between resident organisms, and affording some protection against perturbations/lethal agents in the bulk phase.

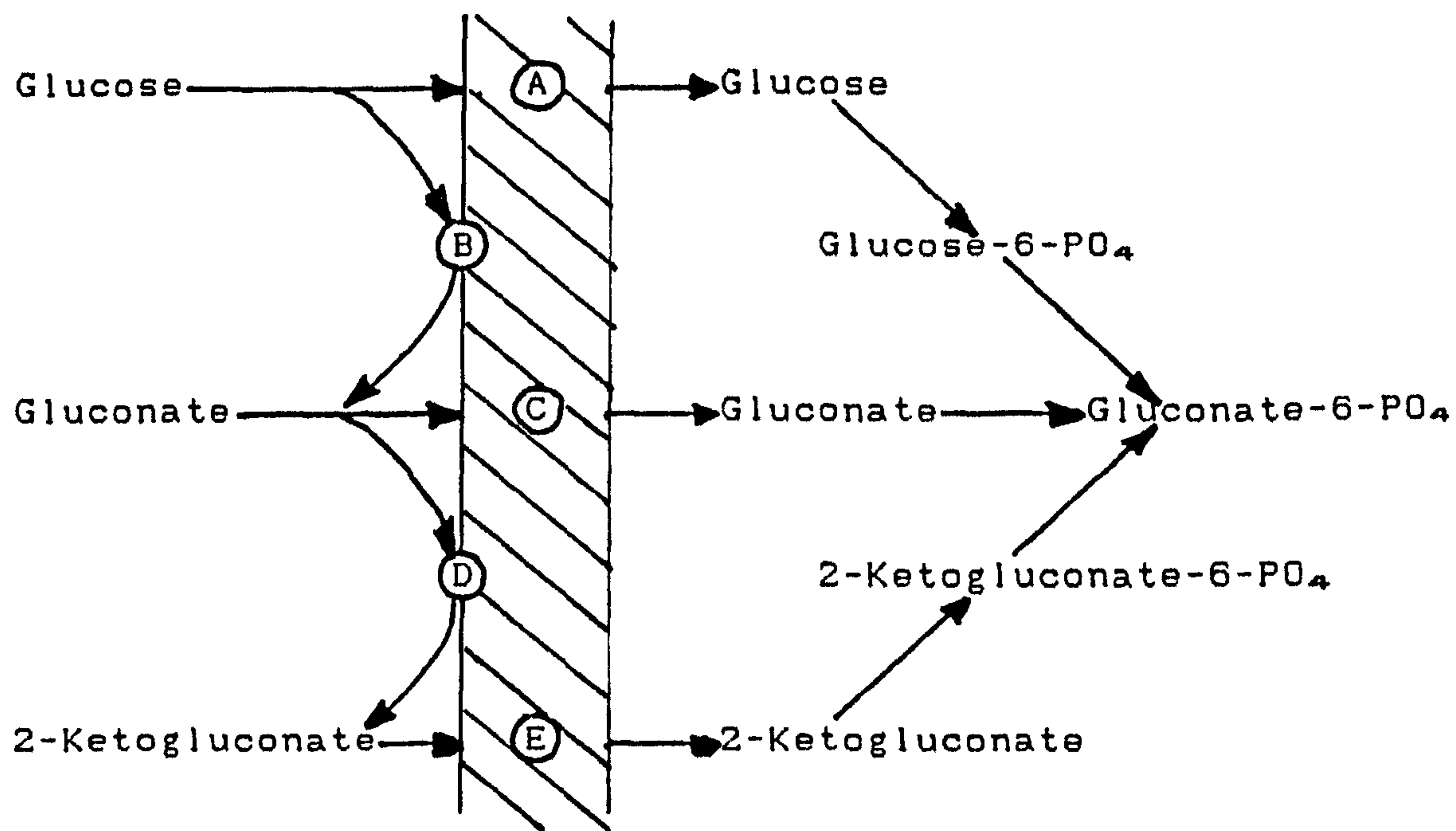
7. Uptake of glucose by *Pseudomonas fluorescens*

The Entner-Doudoroff pathway appears to be the major route for glucose catabolism in *Pseudomonas* (Lynch and Franklin, 1977). There are three possible routes by which glucose may enter the Entner-Doudoroff pathway in *P. fluorescens* (Eisenberg et al, 1974), (and also *P. aeruginosa* and *P. putida* (Lynch and Franklin, 1978)). These three routes are shown on Fig 1.3.

1.3 Pathways available for glucose catabolism in
Pseudomonas fluorescens

Key to symbols:

- A glucose uptake
- B glucose dehydrogenase
- C gluconate uptake
- D gluconate dehydrogenase
- E 2-ketogluconate uptake



The first route involves the uptake of glucose followed by phosphorylation and oxidation to 6-phosphogluconate (6-PG). The second involves direct oxidation of glucose to form extracellular gluconate, and the subsequent uptake and phosphorylation of gluconate to yield 6-PG. Finally, gluconate formed from glucose oxidation can be further oxidised to form extracellular 2-ketogluconate (2-KG), which is then taken up, phosphorylated, and reduced to 6-PG.

Lynch and Franklin (1978) reported that the synthesis and (or) activity of the three uptake systems appeared to be regulated to favour the utilization of glucose or gluconate by the direct oxidative non-phosphorylated pathway through 2-KG as an intermediate at low growth temperatures (5°C). However, at higher growth temperatures (eg 30°C) the major route for glucose or gluconate catabolism appears to be through the direct uptake and phosphorylation of these substrates. The differential effect of temperature on the activities for uptake of glucose, gluconate, and 2-KG, the lack of coordinate induction for the three uptake systems, and the lack of competition in the initial uptake rates between the three substrates shown in this study demonstrated the presence of independent uptake systems for glucose, gluconate and 2-KG.

It has been suggested that the utilisation of glucose by P. aeruginosa is dependent on an inducible glucose transport system which may play a regulatory role in glucose metabolism (Branford White et al, 1977a). It has been demonstrated in P. aeruginosa that a high- and low-affinity system exist (Branford White et al, 1977a). When the glucose supply is limited P. aeruginosa alters its metabolism in order to

facilitate utilization of glucose.

8. The approach adopted in this study

The initial aims of this project were diverse, and were designed to give a greater insight into the factors affecting development of biofilm which was subjected to fluid flow: the primary aim of the project was to study the effect of various flow rates and nutrient conditions on bacterial biofilms.

The approach adopted was as follows:

(i) Initially biofilms were investigated in the absence of fluid flow. Various bacterial species and substrates were used.

(ii) Following this, a considerable period was spent setting up the experimental methods necessary for establishment and analysis of bacterial biofilms subjected to flow in the flow rig. This included establishing techniques for preattachment of biofilms, determination of numbers of attached cells at the start and end of each run, and measurement of substrate uptake.

(iii) Experiments were then carried out in which detachment of cells and uptake of glucose over time were measured for biofilms which were subjected to various flow rates and nutrient conditions in the flow rig. The results were compared with results of experiments using freely suspended cells.

CHAPTER TWO

MATERIALS AND METHODS USED IN THIS STUDY

1. Introduction

The setting up of the experimental rigs, and development of techniques for measuring specific parameters during an experimental run was a time-consuming part of this project. The development work is described in this chapter.

2. Maintenance and growth of stock cultures

Stock cultures were kept in a broth medium (PYE) and in glucose medium. The PYE medium contained 0.1% (w/v) peptone and 0.07% (w/v) yeast extract powder in distilled water, adjusted to pH 7.4 with 1M NaOH (Fletcher, 1976).

The glucose medium contained 0.544g KH_2PO_4 (adjusted to pH 7.4 using NaOH), 0.2g glucose, 0.38g NH_4Cl , and 0.6 ml of a salt solution, made up to 100 ml using distilled water. The salt solution contained (l^{-1}) 10 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.4 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The KH_2PO_4 solution was sterilised by autoclaving (121°C , 15 mins) in a 250 ml side-arm flask: the glucose and NH_4Cl solutions were autoclaved separately (121°C , 15 mins) and added aseptically. The salt solution was filter sterilised using a 0.45μ porosity cellulose acetate filter, and was then added to the flask, making the total medium volume up to 100 ml.

In some experiments, the glucose medium described above was supplemented with 0.2 g of galactose, gelatin or glycerol per 100 ml. These were chosen for the following reasons: galactose because it is a complex sugar, gelatin as it is a

protein, and glycerol because in a previous study (McEldowney and Fletcher, 1985) it had been shown to affect attachment of some species considerably.

After growth for 24 hours at 15°C on a rotary shaker at 150 rpm, stock cultures were maintained at 4°C for 3 weeks in 250 ml side-arm flasks. Agar slopes and agar plates were made up by adding 0.75% agar to the PYE medium. Slopes of the cultures were kept at 4°C and were subcultured every 3 months. Growth was followed turbidometrically at 540 nm with a Corning colorimeter 252. Experiments were carried out at 15°C, so that the results were comparable with those of others working in the group.

3. Preliminary experiments: the selection of a suitable organism and medium for use in the test rig

3.1 Introduction

While the flow rig was being built, studies were carried out using 5 organisms and a selection of media and attachment surfaces, in order to characterise the attachment properties of the 5 species under various conditions, and enable selection of a suitable species for use in the test rig.

The species considered included Acinetobacter calcoaceticus, Escherichia coli, Pseudomonas fluorescens, an Enterobacter sp., and a Flexibacter sp.. These species were all isolated from a polythene sheet following immersion in fast running water at Baginton Weir, River Sowe, Coventry (full details in Pringle et al, 1983b), and were all gram negative. The growth and attachment properties of each organism were investigated.

3.2 Surfaces considered

The surfaces used in these assays were polystyrene petri dishes (PD) (Sterilin Ltd., Teddington, England), tissue culture treated polystyrene (TCD) (Costar, Cambridge, Mass.) and coupons of stainless steel. The stainless steel coupons were cleaned by sonication in distilled water for 5 mins, and then rinsed 5 times in distilled water. The coupons were then whirlmixed for 1 min in a small bottle containing chloroform, and then rinsed 5 times in distilled water. The coupons were laid on a clean glass surface and sterilised in a dry air oven at 160°C for 2 hours.

3.3 Growth and attachment of cells

After inoculation with 1 ml of a stationary phase culture, cultures were grown in the test media for 24 h (unless otherwise specified), at 15°C on a rotary incubator at 150 rpm, before sampling. The cultures were then harvested by centrifugation (15 min, 10,000 g_{av}, 15°C). The cells were washed once in 0.01 M HEPES buffer, pH 7.4, and resuspended in the same buffer at various pH values, or in the medium used during the growth stage, to an absorbance of 0.3 (measured at 540 nm using Corning Colorimeter 252), corresponding to 1.7×10^7 cells/ml. Aliquots of 5 ml of the cell suspension were pipetted into the two types of petri dish used. The stainless steel coupons were placed in a petri dish and 5 ml of the cell suspension added. After a specified time, the petri dishes were washed 3 times with distilled water to remove loosely attached bacteria, and the remaining bacteria were then fixed with Bouins fixative (71% v/v saturated aqueous picric acid, 24% v/v formalin, 5% v/v

acetic acid). The cells attached to the petri dishes were stained with crystal violet at a concentration of 5 g/l (Fletcher, 1976; 1977), and the level of bacterial attachment was estimated indirectly by measuring absorbance at 590 nm on a spectrophotometer (Unicam Instruments, Cambridge). Four readings of randomly selected areas were taken from each of the duplicate dishes. Cells attached to the stainless steel surfaces were stained using acridine orange (0.01 g/l) and the stainless steel discs were attached to glass microscope slides using double sided sellotape. The numbers attached were counted using an epifluorescent microscope fitted with a blue filter at a magnification of x400. A defined area of 25 x 125 μm was counted in 40 different fields, and the results expressed on a unit area basis.

The following parameters were investigated:

- (i) Attachment of different bacterial species.
- (ii) The effect of growth and attachment in different media.
- (iii) The effect of varying attachment incubation time.
- (vi) The effect of three different surfaces on attachment level.
- (v) The effect of culture age on attachment.
- (vi) The effect of changing the pH of the growth medium, and/or the pH during attachment.
- (vii) The effect of varying temperature of incubation during the attachment stage.

Many of investigations were similar to those carried out by McEldowney (1984), and the results were in general consistent with the previous study.

3.4 Results

Figs 2.1-2.4 show the change in attachment level with time for Pseudomonas fluorescens cells which were grown up and attached in different media to PD and TCD surfaces. It can be seen that the cells grown up and attached in the glucose-galactose medium showed the highest level of attachment. Attachment to PDs was generally greater than attachment to TCDs which supports observations made by McEldowney and Fletcher (1985). However, McEldowney and Fletcher also showed that cells grown and attached in the medium containing glycerol showed a much greater level of attachment. This was not borne out in the present study.

Fig 2.5 shows the effect of culture age on the attachment of Pseudomonas fluorescens to PD and TCD surfaces. The culture was grown up for 22, 33 or 72 hours before the start of the attachment assay. It can be seen that the medium in which the cells were grown and attached, the surface and the culture age all affect the attachment level, and demonstrates the importance of ensuring that the conditions are constant in any given series of related experiments.

Figs 2.6 and 2.7 show the effect of pH and temperature on the attachment of Pseudomonas fluorescens. It was difficult to reproduce the pH experiments: results tended to vary from experiment to experiment. This was also observed by Duddridge for several organisms, including Pseudomonas fluorescens (personal communication).

Only a small selection of representative results are given in this section. This is because the majority of these experiments have previously been carried out in other studies (eg McEldowney and Fletcher, 1986a; Fletcher, 1976).

2.1 Change in attachment with length of incubation period for cells grown in glucose medium for Pseudomonas fluorescens cells.

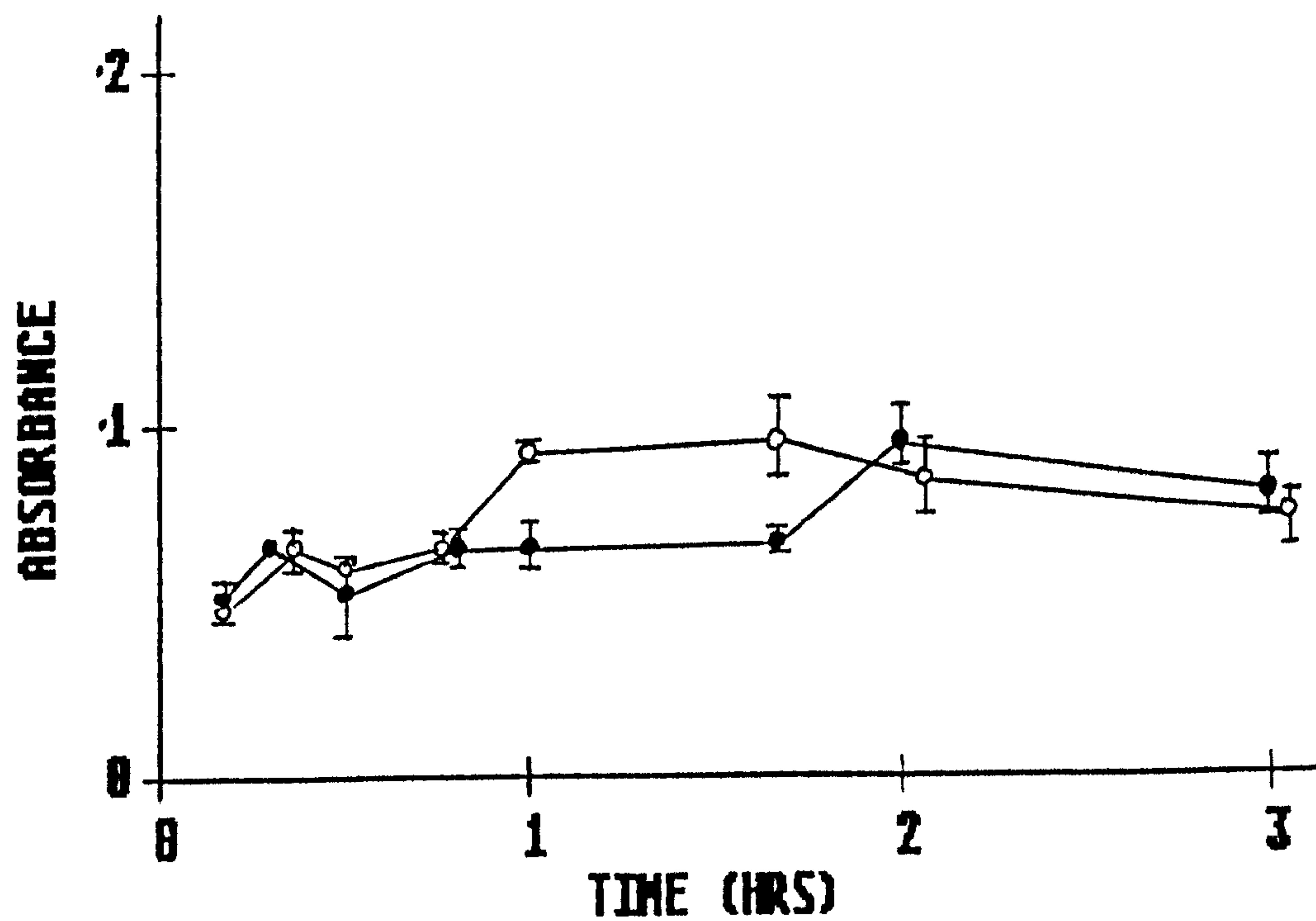
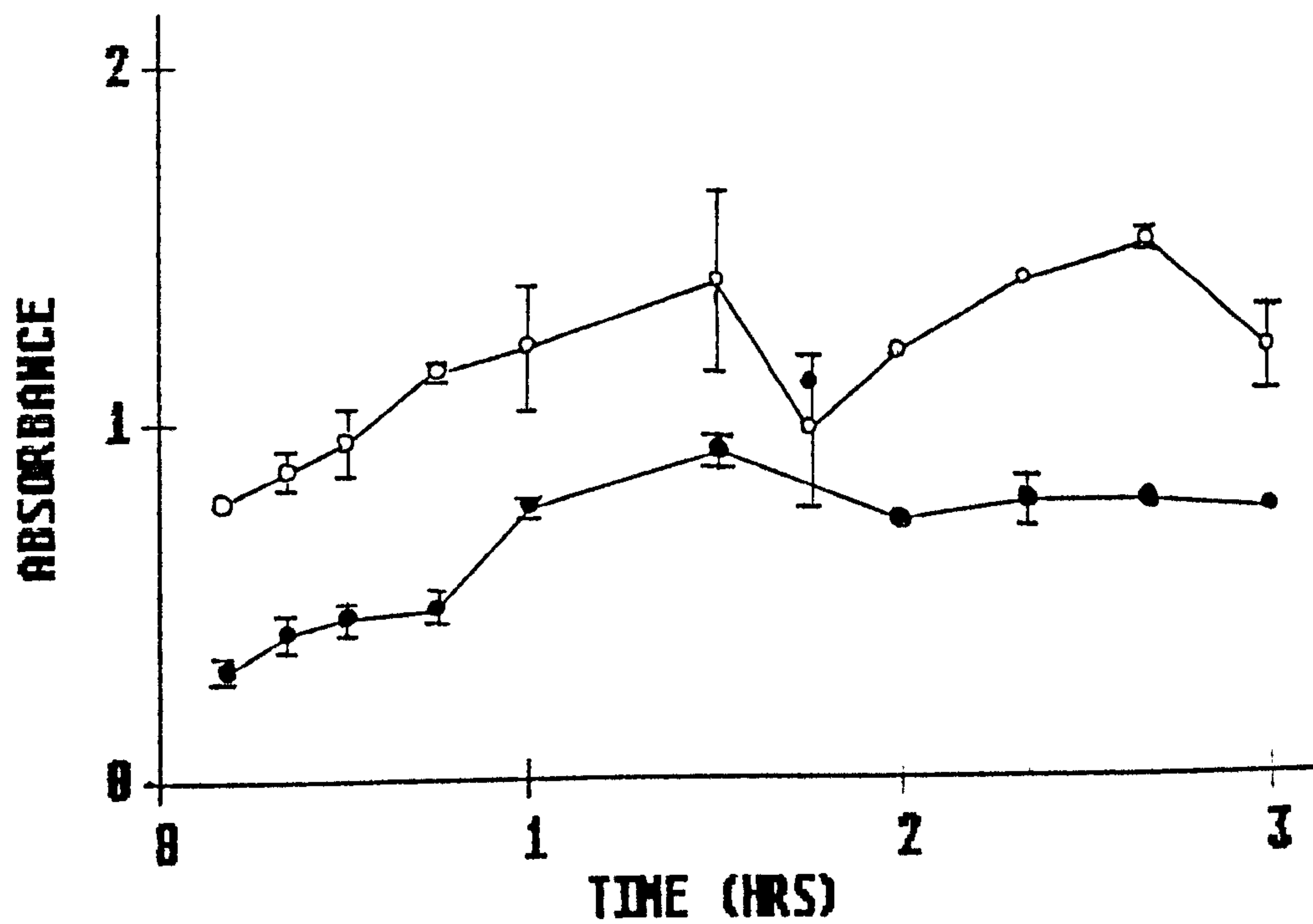
2.2 Change in attachment with length of incubation period for cells grown in glucose-gelatin medium for Pseudomonas fluorescens cells.

Key for both graphs:

○ TCD

● PD

Note: Where no error bar is shown, the point is derived from a single result.



2.3 Change in attachment with length of incubation
period for cells grown in glucose-glycerol
medium for Pseudomonas fluorescens cells.

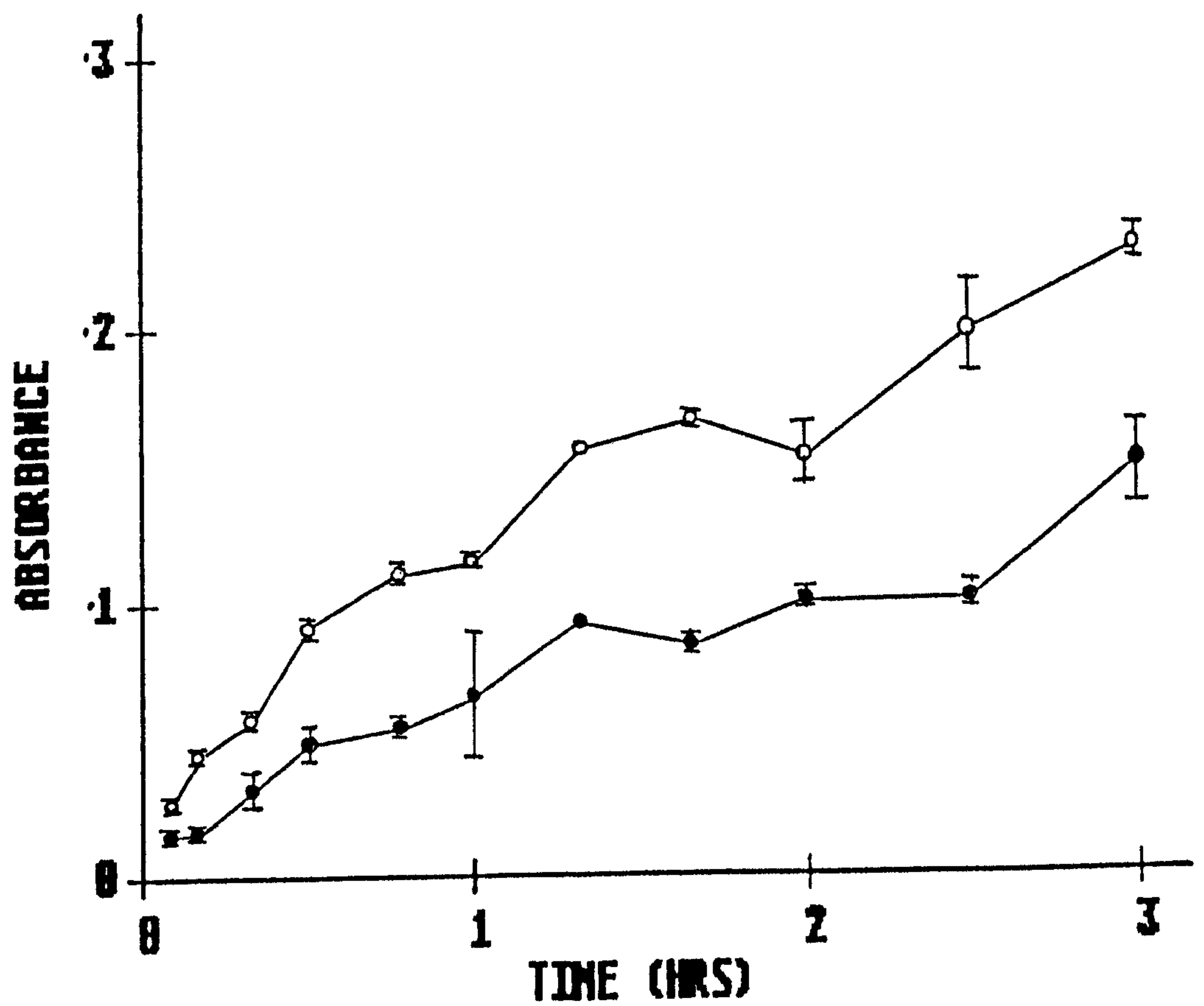
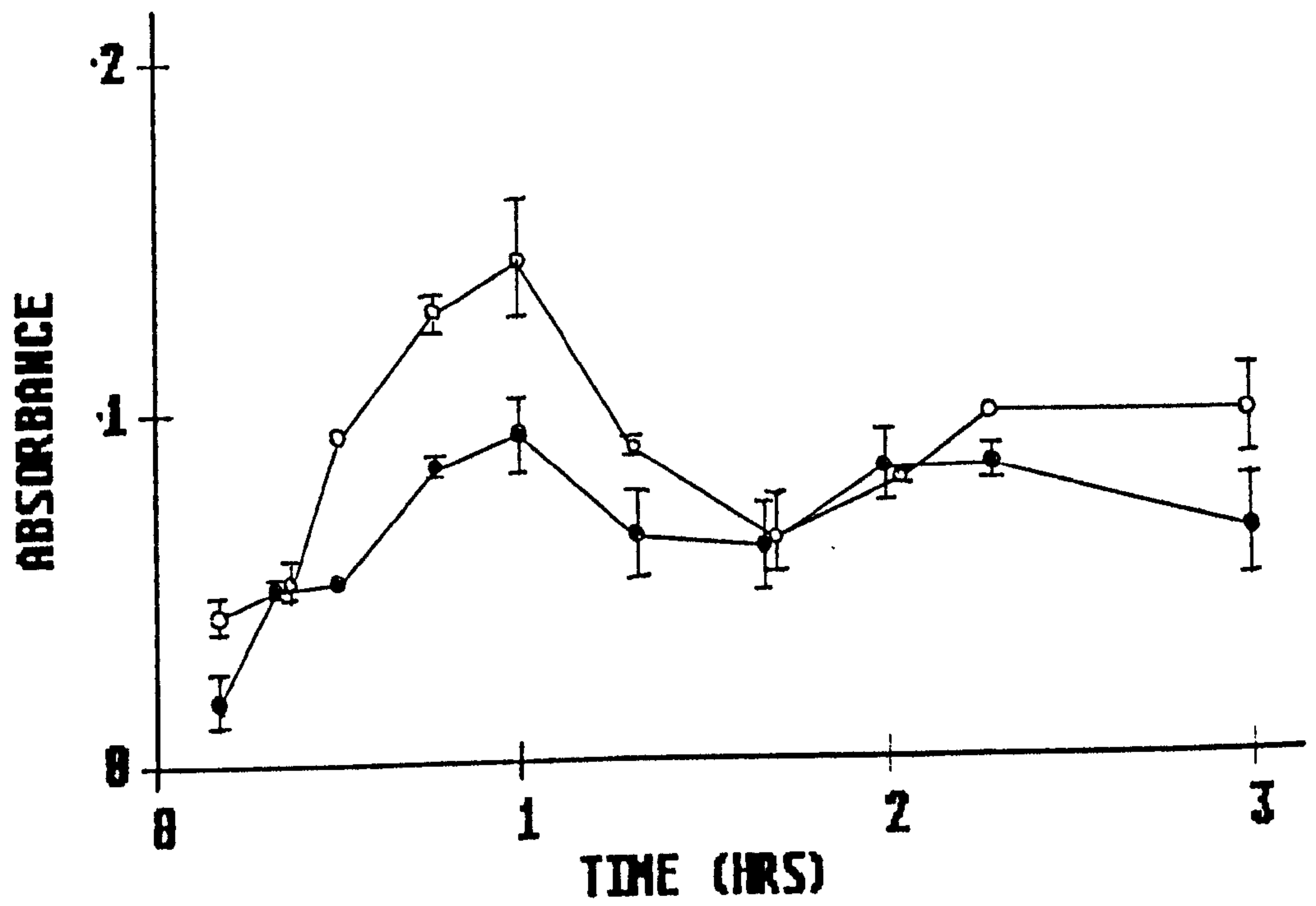
2.4 Change in attachment with length of incubation
period for cells grown in glucose-galactose
medium for Pseudomonas fluorescens cells.

Key for both graphs:

○ TCD

● PD

Note: Where no error bar is shown, the point is derived
from a single result.



2.5 Effect of culture age on attachment for
 Pseudomonas fluorescens cells.

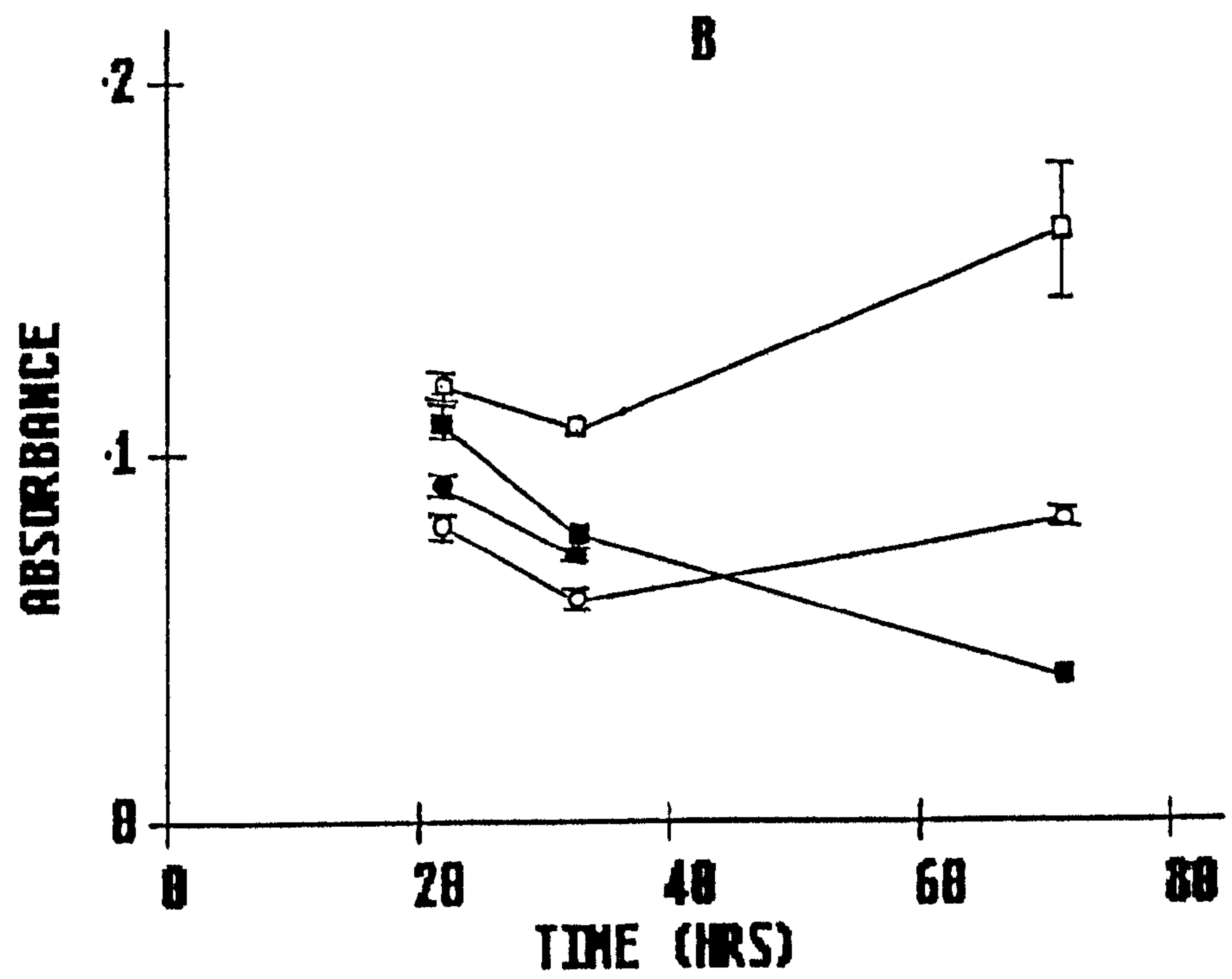
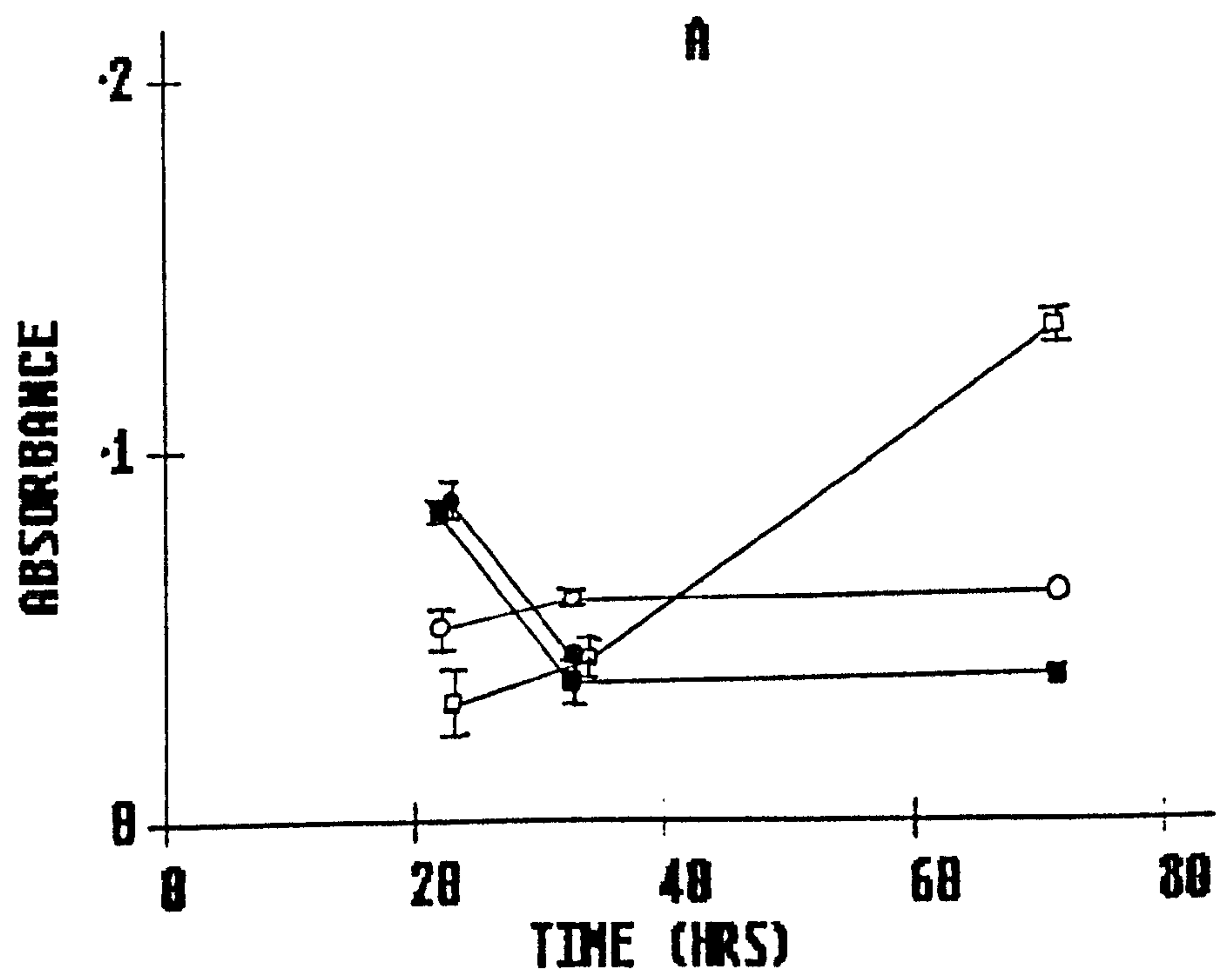
Cells grown in:

- Glucose medium
- Glucose-galactose medium
- Glucose-gelatin medium
- Glucose-glycerol medium

Graph A shows attachment to PD

Graph B shows attachment to TCD

Note: Where no error bar is shown, the point is derived from a single result.



2.6 Attachment of Pseudomonas fluorescens cells which were grown up and attached in glucose medium at various pH values.

Key:

○ TCD

● PD

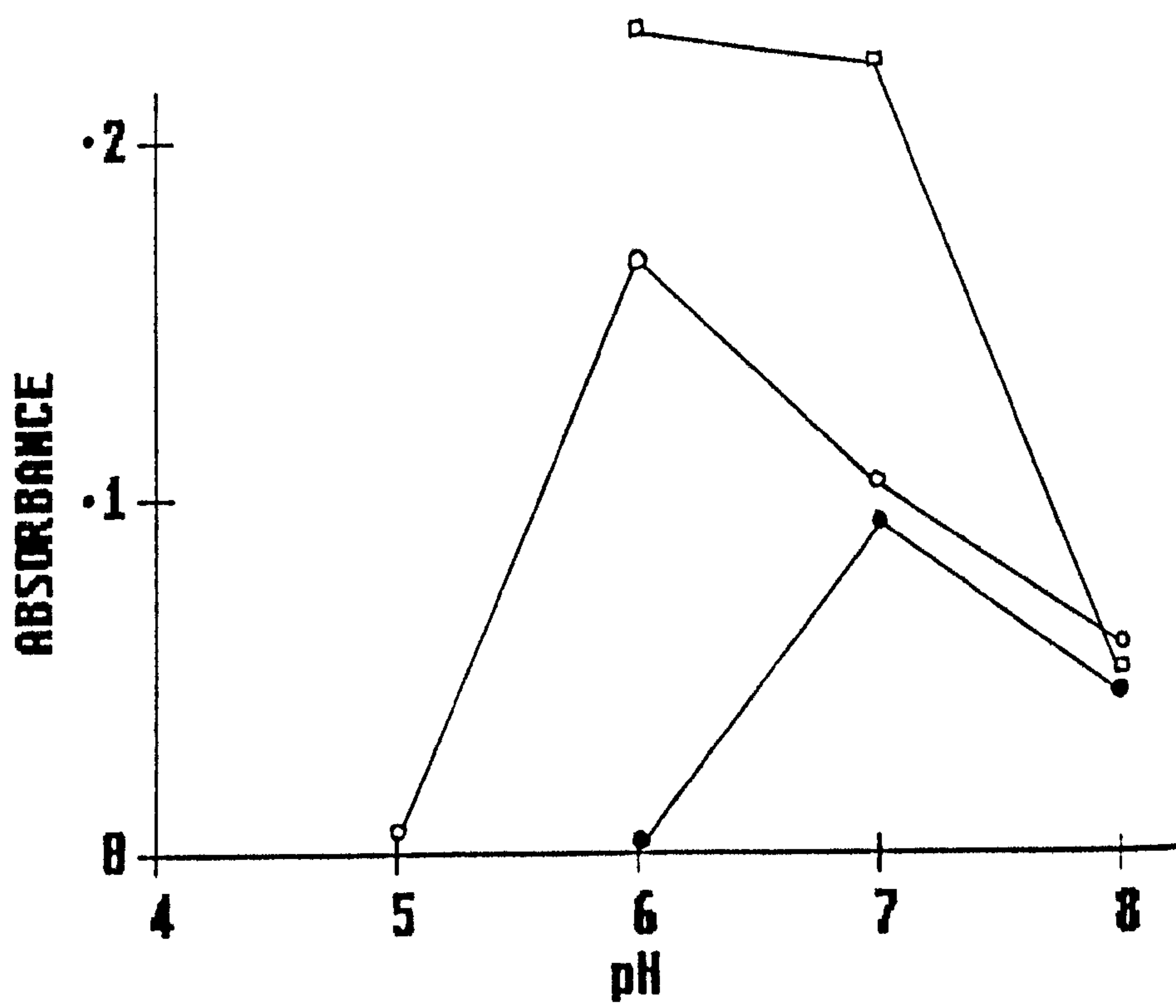
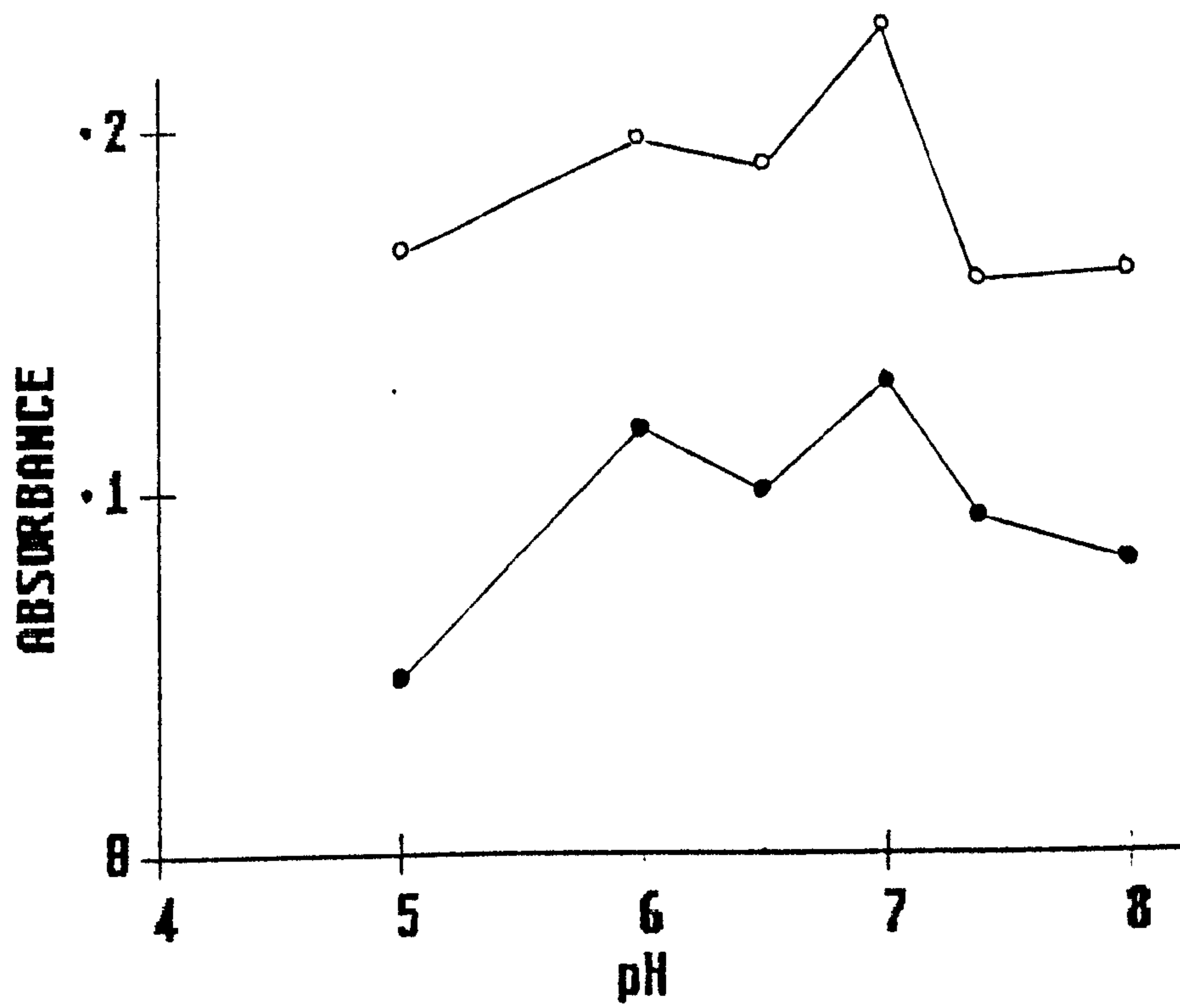
2.7 Effect of temperature during the attachment incubation period on Pseudomonas fluorescens. Cells grown up and attached in glucose medium at various pH values to tissue culture dishes (TCDs).

Key:

● 4 °C

○ 15 °C

□ 25 °C



3.5 General conclusions

The Acinetobacter sp. used grew on very few substrates, so it was decided that this was less suited to experiments using the flow rig, as it was foreseen that several media might eventually be used in the flow studies. The Enterobacter sp. tended to form aggregates in broth culture, and cells attached to surfaces were also observed to be in clumps, hence this species was less suitable for the subsequent studies as microscopic observation and counting of numbers attached would be more difficult. The Flexibacter sp. was eliminated as this is a gliding bacterium, and this could have created difficulties in the analysis of future experiments.

The Pseudomonas fluorescens sp. was eventually chosen as being the most suitable organism: the biofilms did not show aggregation, and could be easily observed microscopically. It grew on a wide range of carbon sources.

The cells were grown up in glucose medium (prepared as described in Section 2, page 33) for the experiments using the flow rig. In addition to being a chemically defined medium, glucose was selected as substrate because it does not adsorb to surfaces, it is a biologically important and common simple carbon source, and is a central metabolite that is normally metabolised by constitutive enzyme systems (Caldwell and Lawrence, 1986).

4. The Flow Rig

4.1 Description of the Flow Rig

Small scale experimental flow rigs were built specially for this project at Harwell Laboratory. They were modified

and scaled down versions of apparatus used at Harwell. The small size meant that the rigs could be fitted into an autoclave and sterilized between experimental runs, and therefore could be used under sterile rather than hygienic conditions. In addition, the small size made the apparatus more suitable for the use of techniques such as tracer studies.



The experimental rig (Figs 2.8 to 2.10) consisted of three vertical 'legs' in parallel, each containing a test section, in which cryogenic stainless steel tubing could be inserted and removed. The flow through each test section was controlled by means of a flow meter (In-line Gapmeter Type GS 3/8"S, Platon Flowbits Ltd., Basingstoke, England) and diaphragm valve (1/4" Type 50034 Diaphragm, Bioengineering U.K. Ltd., Caterham, England) located beneath the test section. If necessary, each 'leg' of the rig could be isolated during a run by closing off the valves at the top and bottom of the leg. The test section could then be drained by means of an outlet valve, enabling the stainless steel tubes to be removed for analysis.

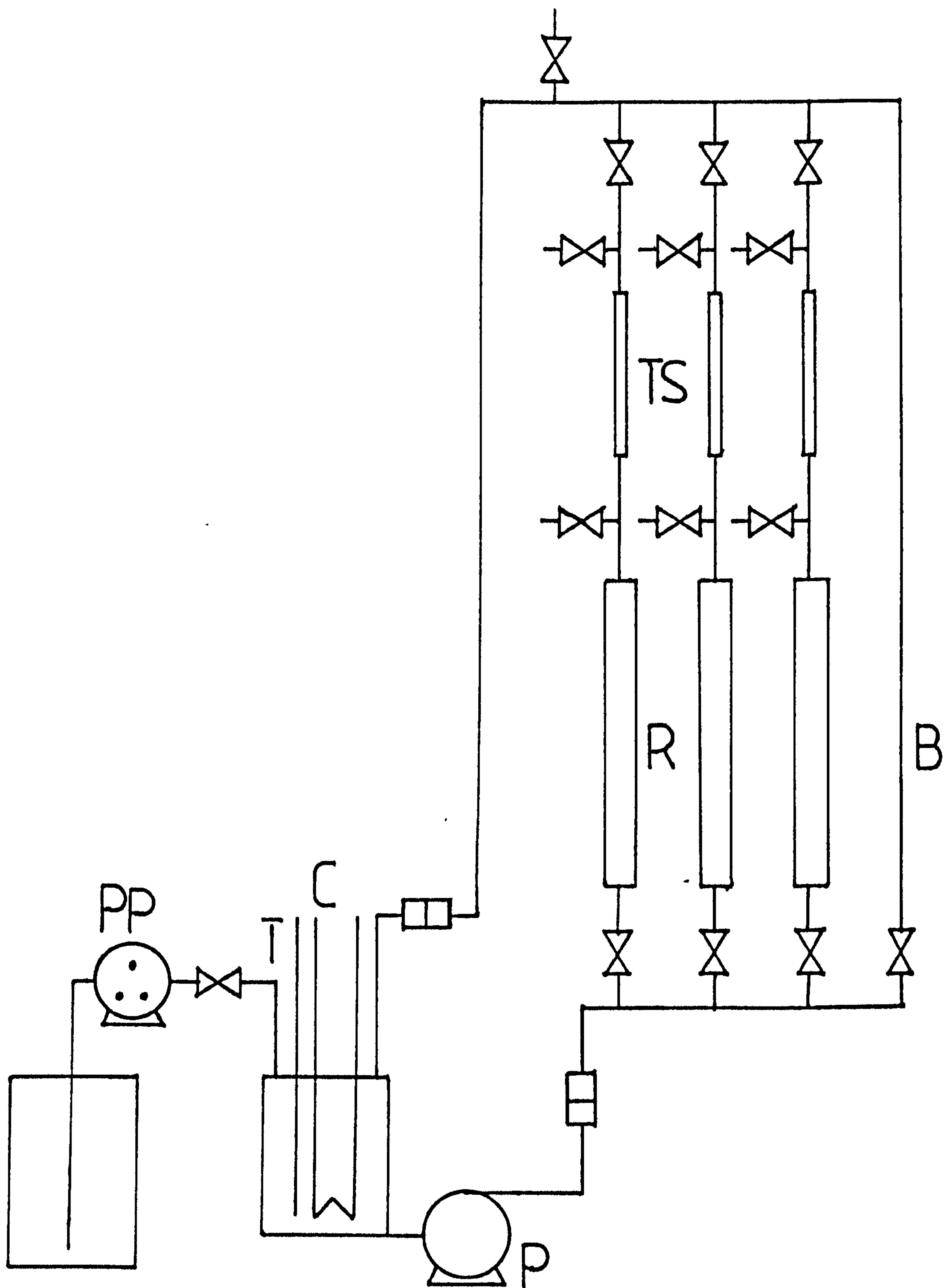
Flow rates between 0.2 and 4.4 l/min were obtainable, corresponding to a range of average velocities through the test sections of 0.118 to 2.597 m/s. A bypass line, also controlled by a diaphragm valve (1/2" Type 50108 Diaphragm, Bioengineering U.K. Ltd., Caterham, England), was fitted.

Fluid was continuously recycled around the rig and through the test sections by means of a centrifugal pump (Magnetically coupled centrifugal pump, Type EMP 50/7, TEP Ltd., Cadnam, Hants.). A specially adapted stainless steel fermenter vessel (LH 0.7 l fermenter vessel) served as a

2.8 Diagram of the flow rig.

Key:

B	Bypass line
C	Cooling coil
P	Centrifugal pump
PP	Peristaltic pump
R	Rotameter
T	Thermometer
TS	Test section
	Valve
	'Quick Release' coupling.



AS ORIGINAL

2.9 Photograph of the flow rig.

**PAGE
MISSING
IN
ORIGINAL**

2.10 Photograph of the flow rig.

**PAGE
MISSING
IN
ORIGINAL**

recycle tank. The total rig volume was ~1.2 l. The lid of this was detachable and was fitted with 6 ports. Two of these were used for the recycle line from the test sections and for a medium feed line for filling the rig. In addition a port through which small quantities of material could be added to the circulating medium during a run was fitted. One of the three remaining ports was used to hold a thermometer and the other two were used to insert a cooling coil into the fermenter vessel. The cooling coil was connected with lagged rubber tubing to a Churchill Chiller Thermo Circulator (Churchill Ltd., England). At one stage a cooling finger, which used only one port, and a temperature sensor and heater were fitted in an attempt to automate the temperature control in the rig: however this was unsuccessful as the cooling finger, being of a small surface area, was not sufficient to maintain the required temperature.

It was possible to run the rig as a fermenter, and allow a culture to grow at the same time as studying its attachment. In fact this was never done, due to the difficulties in data interpretation. In addition, fouling on all the surfaces of the rig would also have occurred on an increased scale.

The basic procedure adopted for this study was to preattach a monolayer of cells to the inside of the stainless steel tubes used as test sections, and then insert the tubes into the rig. This meant that the experiments were conducted using a well defined population, and the problems due to fouling in the rig were minimal.

The rig was constructed to take test sections of cryogenic stainless steel, internal diameter 0.6 cm, and length 15 cm. These were held in place in the rig by silicone

tubing of 0.6 cm internal diameter, and hose clips. The wall thickness of the stainless steel tubes was negligible ($\ll 1$ mm), so there were no significant constriction or expansion effects at the junction between the silicone tubing and stainless steel tubes which would have affected the hydrodynamic flow conditions. The 15 cm lengths of stainless steel tube were found to be unnecessarily lengthy, and cumbersome to manipulate, so they were cut down to 7.5 cm, and the silicone tubing holding them in place was replaced by longer lengths. Subsequently, in radiotracer experiments the length of the stainless steel test sections was again reduced to 5 cm, to increase the convenience of manipulation still further, and reduce the amount of tissue solubiliser required to remove attached cells from the tubes. This allowed two test sections to be fitted in each leg of the rig. Later it was found that three 5 cm sections could be fitted in, and in the final series of experiments this arrangement was used.

4.2 Sterilisation of the Flow Rig

To enable the flow rig to be autoclaved it could be separated into two sections by means of two quick-release couplings (Hansen Couplings, Series 4-ST, Guyson International, Otley, England). The magnetically driven centrifugal pump motor could be unscrewed from the pump head, which contained the impeller, the pump head forming a permanent section of the flow rig circulation system.

After removing the pump motor, the rig was then split into two sections using the quick-release couplings. The open ends of the couplings were covered with foil, and the medium feed line and overflow pipe were closed off with adjustable

clips and the ends protected with foil. The two sections were autoclaved at 110°C for 30 minutes. (It was possible that the rig could have been damaged if it had been autoclaved at 121°C, as several materials were used in its construction, and differential expansion may have resulted in the cracking of some components.) After cooling, the foil was removed from the quick release couplings, and the open ends were flamed, before fitting the two sections of the rig together.

4.3 Rig cleaning procedures

After a run the rig was completely filled with 0.2% Lipsol detergent (LIP Equipment and Services, Shipley, England) or with 0.2% Micro detergent (International Products Corporation, Chislehurst, Kent), and left overnight. Following this, the rig was drained and rinsed in at least 50 rig volumes of tap water, drained, and then rinsed with at least 5 rig volumes of distilled water.

5. Procedure used during an experimental run

The procedure originally used is described here. This has undergone several modifications over time, which are set out later in this chapter. The first experiments using the rig involved pre-attaching a monolayer of cells to the inside of the test sections, rinsing the test sections to remove loosely attached cells, placing the test sections in the flow rig, and testing the percentage detachment from the tubes at various flow rates.

5.1 Procedure for cleaning the test sections

The tubes were cut to the required length, and the ends sanded using an orbital sander to remove any burrs of metal. In the first instance the stainless steel tubes were prepared

by:-

(i) Soaking in chloroform for 1 hour to remove grease, followed by 5 rinses in distilled water.

(ii) Sonicating for 5 minutes in a sonicating water bath (Pulsatron 125, Kerry Ultrasonics Ltd., Hitchin, Herts.), followed by 5 rinses in distilled water.

(iii) sterilising in a hot air oven at 160°C for 2 hours.

5.2 Preattachment of a monolayer of cells to the inside surface of the test sections - the 'attachment incubation'

Cells which had been grown for 24 hours, to early stationary phase (the optical density was approximately 0.7, measured at 540 nm using a Corning Colorimeter 252) were washed once in 0.04 M KH_2PO_4 buffer (adjusted to pH 7.4 using 1 M NaOH) and resuspended in this buffer to an optical density of 0.3 using a colorimeter (Corning Colorimeter 252). A 0.6 cm diameter rubber bung was placed in the one end of a 7.5 cm length of stainless steel test section, which was held vertically, and filled with the resuspended culture. A second bung was then placed in the top end of the tube, and any excess culture wiped off the outside of the tube. A series of tubes prepared in this way were placed horizontally in a flat metal container, such that they could roll freely. This was important in order to prevent sedimentation of cells in the tube. The container was then placed in an orbital shaker rotating at 110 rpm, 15°C, and left for 1 hour. They were then removed and rinsed 3 times in distilled water. Some tubes were placed in the rig and others were used as controls.

At the start, an attachment incubation time of 60 mins appeared satisfactory: after this period a reasonably evenly

spread thin monolayer of cells had attached, although there were some clumps of cells. The average number of cells attached was approximately 1.6×10^4 cells/mm² (ie 10 cells per field of 625 μm^2).

5.3 Preparation of the rig at the start of the run and procedure during the run

The flow rig was cleaned and sterilised as described above. Before a run, while the stainless steel test sections were being prepared, the two sections of the rig were fitted together and the pump motor attached. The medium feed line was fitted through the head of a peristaltic pump, and the end was placed in a bottle containing 1 l sterile medium. The clip on the overflow pipe at the top of the rig was loosened, to allow medium to be pumped into the rig. The medium was pumped in until it reached the top of the flow meters, and when the test sections, with their pre-attached monolayer, were placed in the rig, the rig was filled with the remainder of the medium. The rig was filled in two stages to minimise the time period between the attachment stage, in order to subject the attached bacteria to as little change as possible.

The valves below the flow meters in each leg of the rig were then closed off, and the valve controlling flow through the bypass pipe was set to the fully open position. The circulation pump was then switched on, and the flow rate through each test section was set by gradually increasing the flow through the test sections. In the first test runs, it was found that bubbles were a problem, particularly during the early stages of a run. It was likely that there could be

some stripping of the attached cells due to the bubbles, and so two adjustments in the procedure helped to eliminate them:-

(i) The quantity of medium supplied to the rig was increased to 1.1 l. This reduced the amount of air in the rig from ~ 200 ml to ~ 100 ml.

(ii) When setting the flow rate through each leg of the rig, it was found that the valves controlling the flow needed to be opened very gradually, while simultaneously closing the valve in the bypass pipe at an approximately equal rate.

The start of the experiment was taken as being the time at which the required flow rate was achieved in each leg of the rig. The run period was not always the same from experiment to experiment, and within any one experiment it was possible to shut down one test section at a time, by closing off the valve at the top and bottom of the individual leg of the rig, and then draining the test section by opening the outlet valve which was situated just below the test section. Hence in any one run, the flow rate and the run length could be independently varied in each 'leg' of the rig.

After a specified run time the flow rig was shut down by switching off the circulation pump, and pumping the fluid out of the rig using the peristaltic pump. The stainless steel test sections were then removed from the rig and rinsed three times in distilled water. It took approximately one minute to shut down the flow rig and remove and rinse the test sections.

5.4 Method of counting the numbers of cells attached to the tubes

The attached cells were then fixed using Bouins fixative (71% v/v saturated aqueous picric acid); 24% v/v formalin; 5% v/v acetic acid), and stained with the fluorochrome acridine orange.

The tubes were then cut into three sections using a small hacksaw. Each 2.5 cm section was cut open lengthways using tin snips, and the sections were then carefully opened out using fine tweezers, to avoid damage to the fixed monolayer inside. Having ensured the section was as flat as possible, it was fixed to a glass slide using double sided sellotape. A drop of immersion oil (Cargill Type A Immersion Oil, McCrone Research Associates Ltd., London) was placed on the stainless steel section which was then observed under an epifluorescent microscope (Zeiss Standard 18 Microscope) fitted with a blue filter at a magnification of x 400. The number of cells in a defined area of 25 x 125 µm were counted in 40 fields.

5.5 Calculation of the percentage detachment at various flow rates and run times

The numbers of cells which detached were calculated by comparing the numbers of cells remaining adhered to a tube after being in the flow rig with the average numbers of cells attached to the several other tubes, which had not been placed in the flow rig, but had been fixed and stained immediately after the attachment incubation stage.

$$\text{Percentage detachment} = \left\{ 1 - \frac{\text{no. of cells/tube after experiment}}{\text{initial no. of cells/tube}} \right\} \times 100\%$$

6. Problems encountered with the original experimental procedure

Several experiments were run to test the reproducibility of the methods outlined above. It was found that in general the reproducibility was erratic and sometimes very poor indeed. Not only did the results vary markedly, and to no fixed pattern from experiment to experiment, but also the correlation between the attachment levels in tubes which had been exposed to identical conditions in any one experiment was also low. While counting the numbers of cells attached to the tubes it was noticed that occasional tubes had large clumps of bacteria, and that in other tubes there appeared to be very different levels of attachment at each end of the tube: sometimes a sharp line divided the two sides, with one end of the tube showing much higher attachment levels.

There were several factors which were possibly causing the lack of reproducibility shown in these experiments:-

(i) There was a possibility that the cells were becoming starved of oxygen during the attachment incubation. There was also a chance that the length of the attachment incubation period (1 hour) corresponded to the time at which cells were just beginning to become irreversibly attached to the tubes, and consequently a great deal of variation would occur between tubes. Tests were implemented to check whether these effects were causing problems.

(ii) It was thought possible that the tubes were not totally clean, and small variations in the surface of the tubes could affect attachment levels quite markedly. Therefore a range of cleaning methods were tried, and tested to see how this affected the attachment levels.

(iii) The standard deviations for the microscope counts of numbers of cells adhered were very high, and it was felt that an improvement in the method of counting the numbers of attached cells, or a change of method, might be necessary.

(iv) It was possible that variations between batch cultures could cause fluctuations in the results from experiment to experiment, and therefore growing cells in continuous culture was considered.

These four potential reasons for the lack of reproducibility in the attachment levels were investigated in an attempt to improve the reproducibility of the attachment procedure, and the results of parts (i) to (iii) are detailed below. A chemostat was set up in order to check whether the variability between batch cultures was affecting the results. However, as the other investigations were reasonably successful, cells were not grown in a chemostat for any experiments which involved using the flow rig. Preliminary attachment experiments indicated that the attachment levels for chemostat grown cells were much lower than for cultures grown in batch, and the cells were tended to be smaller.

6.1 The effect of reducing the volume of culture inoculated into the tubes and increasing the incubation time

The attachment method, in which the stainless steel tubes were filled with resuspended bacteria for 1 hour has been described above. It was felt that there was a possibility that the bacteria were in some cases becoming depleted of oxygen during the incubation, so the effect of reducing the inoculum of culture was tested. The total volume of the tubes was 2.12 cm³, which was reduced to approximately 1.84 cm³ by the bungs which were inserted at either end of the tube

during the attachment stage. The effect of using a 1 ml inoculum was tested.

There was a possibility that one hour was a critical time at which some of the cells had started to make firm bonds with the surface, but other bacteria were loosely attached by reversible bonds, causing variation in the attachment levels. A test in which attachment time was lengthened to 1.5 hours was carried out (see Fig 2.11), and it was shown that although attachment levels increased, the results still showed very high standard deviations for duplicate tubes.

Nevertheless, although these measures did not solve the problem of lack of reproducibility, it was decided to use an inoculum of 1 ml rather than filling the tube in future experiments, to ensure that a shortage of oxygen did not occur, and to use an attachment time of 1.5 hours to increase the numbers of cells attached. Shortly afterwards this was increased further to 2 hours following an additional experiment (see Fig 2.12) which showed attachment was optimised at 2 hours.

In addition, as a result of some trials, the absorbance of the culture inoculated into the test sections during the attachment incubation was increased to 0.8 (measured at 540 nm, using a Corning Colorimeter 252), which resulted in greater numbers attaching (see Fig 2.13). This was desirable to increase the accuracy of the results from the flow rig studies. Further increase in the absorbance did not appreciably affect the level of attachment.

2.11 Effect of varying the length of the attachment incubation period: up to 1.5 hours.

Initial culture concentration 1.95×10^7 cells/ml

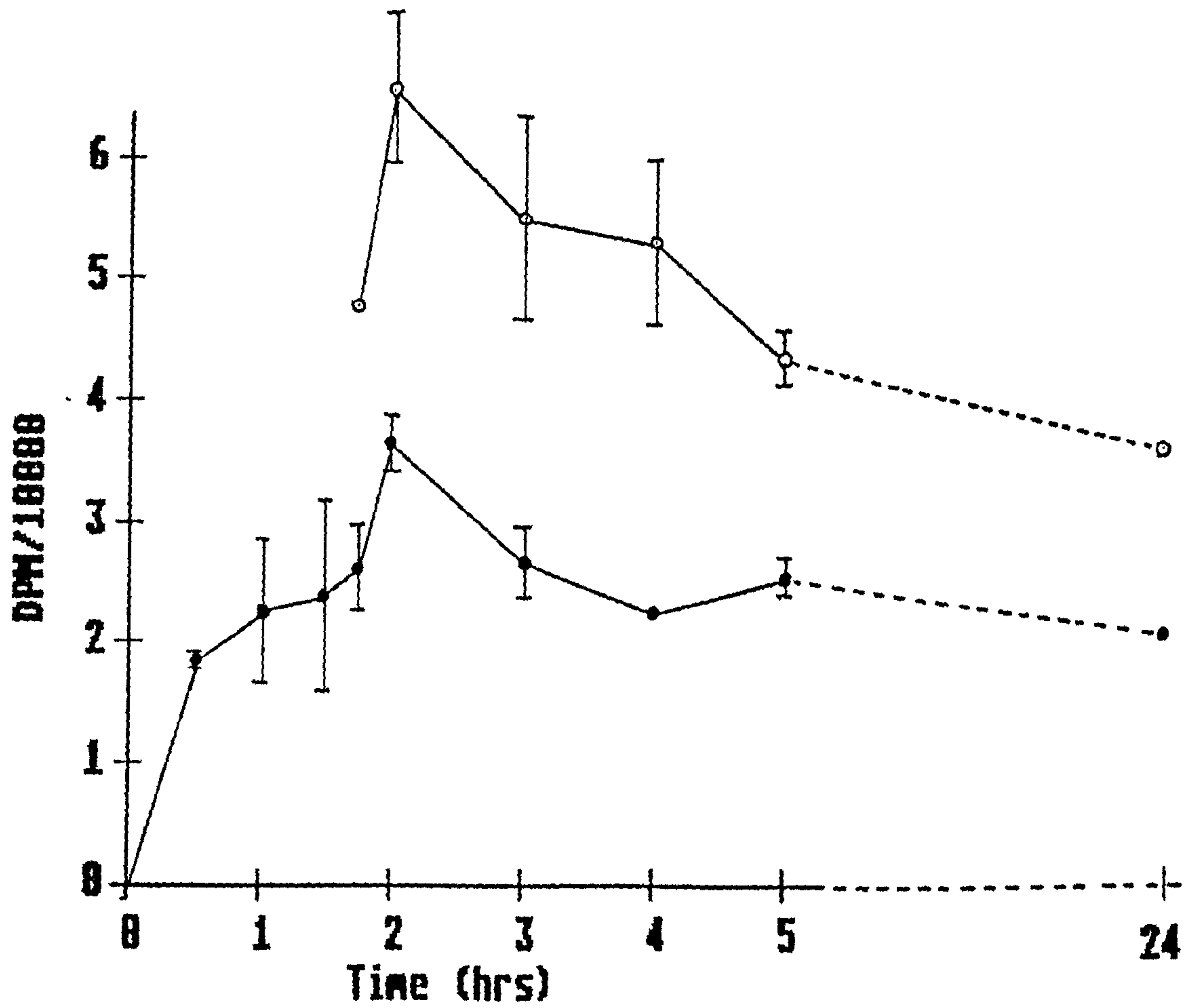
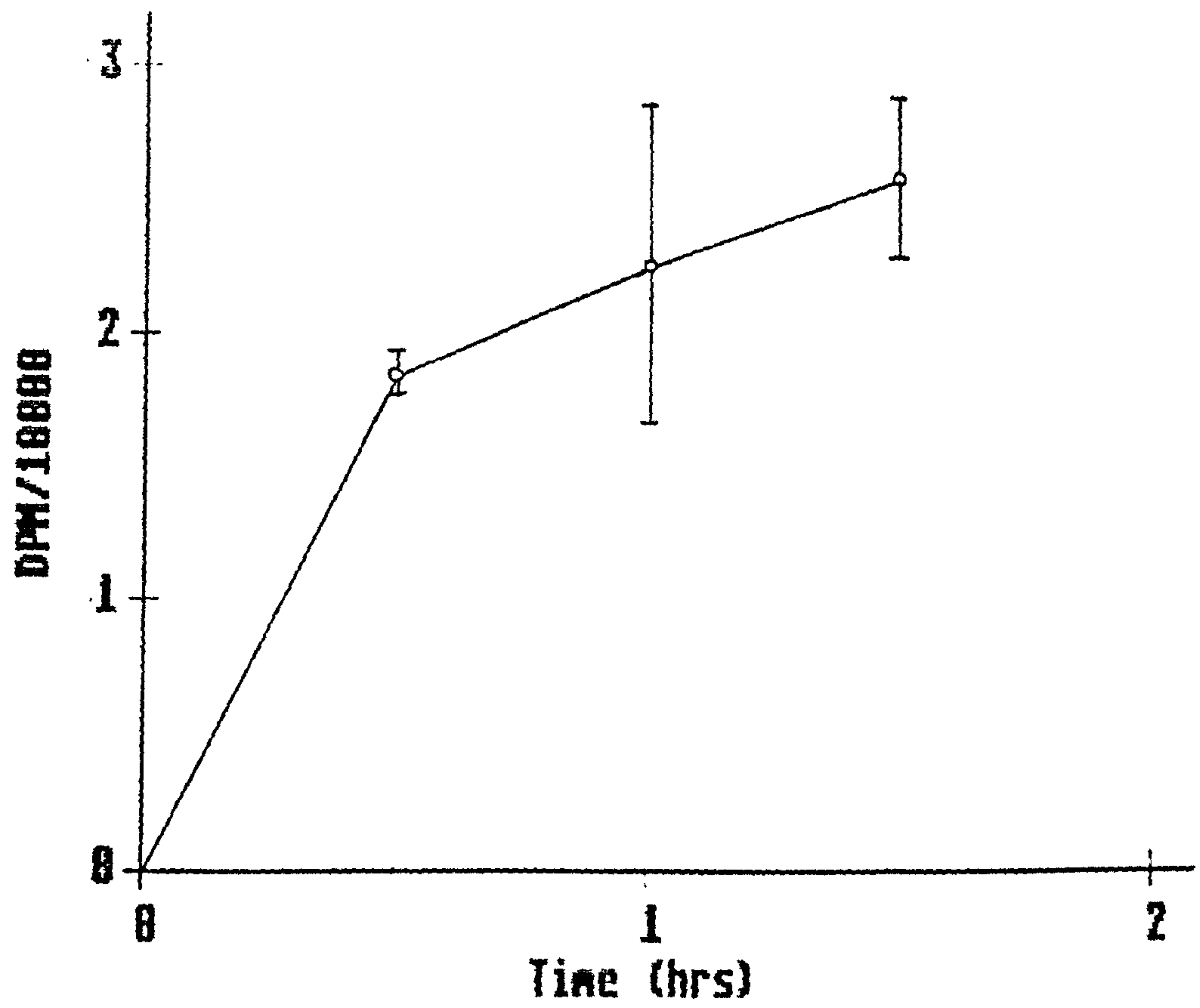
2.12 Effect of varying the length of the attachment incubation period: up to 24 hours.

Key (2.12):

Initial culture concentrations:

- 1.95×10^7 cells/ml
- 4.52×10^7 cells/ml

- Notes:
1. Where no error bar is shown, the point is derived from a single result.
 2. Y-axis: Disintegrations per minute/10,000

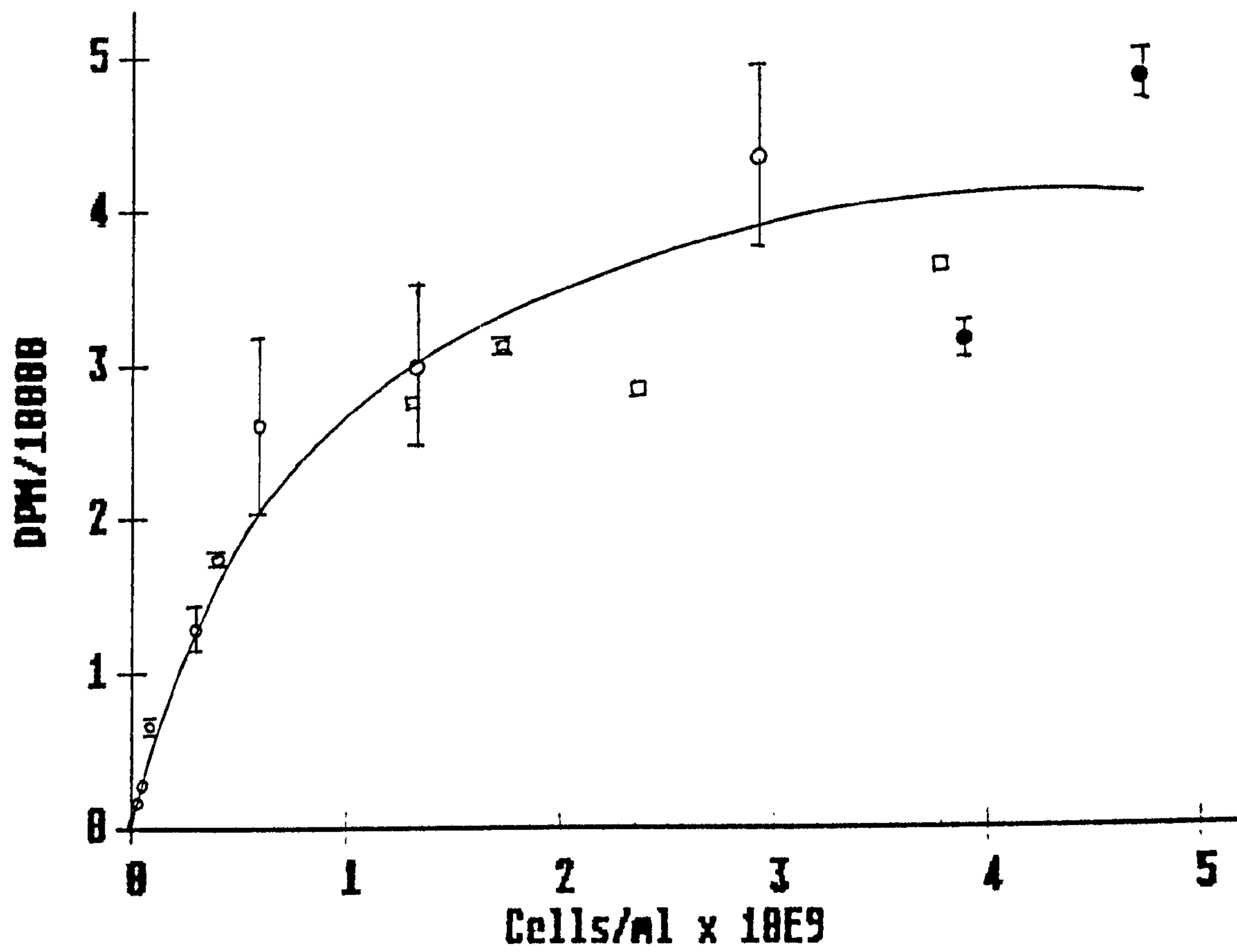


2.13 Effect of cell density on attachment level.

Key:

- Results of 16/7/85
- Results of 16/8/85
- Results of 21/8/85

Note: Where no error bar is shown, the point is derived from a single result.



6.2 The effect of changing the tube cleaning procedure and using a detergent solution during sonication

Various methods of cleaning the tubes were tested in order to check whether the attachment levels were more uniform in tubes which had been cleaned more rigorously, or with alternative detergent solutions. In the original two-stage cleaning procedure (Section 5.1, page 49), the tubes were sonicated in distilled water, and degreased by soaking in chloroform. Several changes to this procedure were made.

As a first attempt at improving the tube cleaning method, the order of the stages was altered, and tubes were sonicated in a detergent solution rather than distilled water. The stainless steel tubes were first degreased by soaking in chloroform and then sonicated in a detergent solution. Particular attention was paid to rinsing the tubes in distilled water many times, then leaving them to soak in distilled water for 24 hours. This was done because Duddridge (personal communication) carried out an experiment in which stainless steel surfaces were soaked in deionised water for periods of 1, 18 and 24 hours after they had been cleaned. A monolayer of cells was then attached to the surfaces and it was found that, the longer the surface had been soaked, fewer cells were detached when the surfaces were subjected to flow. This demonstrated that attachment was affected by the length of the rinsing period, and it was thought that this might be due to the fact that more detergent molecules had desorbed during the longer rinse periods.

The tubes were first soaked for 15 minutes in chloroform, then rinsed 3 times in distilled water, before being

placed in a beaker filled with 1% Micro (International Products Corp., Chislehurst Kent), and sonicated for 5 minutes. The tubes were then immediately rinsed three times with distilled water, and left to soak for 24 hours in distilled water before being rinsed three more times. The tubes were then wrapped in individual foil packets and sterilised for 2 hours at 160°C.

This method of preparing the tubes was tested by attaching cells to the surfaces, as described in Section 5.2, and checking the variability in the numbers attached at the end of the attachment incubation. For a batch of 6 tubes the overall averages were very similar (Table 2.1). However, when this experiment was repeated, there was very poor agreement (Table 2.2).

Further experiments were then carried out to check whether the variability in the levels of attachment could not be reduced. It was decided to:-

(i) Try various detergents other than Micro to check whether this had an effect on attachment.

(ii) To inspect each tube after cleaning. The tube was dipped into a beaker of distilled water and the appearance of the film of water noted on the surface of the inside of the tube. Clean surfaces should have a film of water evenly distributed over the surface, whereas on a dirty surface the film is more patchy.

The first detergents to be tested were Lipsol (LIP Equipment and Services) and Teepol (BDH Chemicals Ltd., Atherstone, Warwicks), at a concentration of 1%. Tubes were also cleaned in Micro, as before, to test whether the use of Lipsol and Teepol improved the cleaning efficiency. The

procedure was slightly modified.

The tubes were soaked for 1 hour in chloroform, and rinsed 3 times in 1% detergent solution. They were placed in beakers with 1% detergent solution, and sonicated for 5 minutes. They were then rinsed twice with 1% detergent solution and sonicated in the same solution for 5 minutes. After this they were rinsed twice in the detergent solution and 20 times in distilled water. They were left to soak for 24 hours in distilled water and then rinsed 10 times in it. At this point the tubes were dipped into a beaker of distilled water and inspected for cleanliness. Each tube was labelled on the outside, and a note made of the observations, in order to try to correlate this with the observed degree of attachment. Uneven or patchy water films were observed in all of the tubes which were cleaned using Teepol detergent, hence the number of cells which attached was tested for only one of these tubes. The tubes were then wrapped in foil and sterilised as before.

A direct correlation between the numbers of cells attached and the observations of films of water in the tubes was observed (Table 2.3). In the case of the three tubes in which the water film was noted to be uneven or patchy, rather than evenly distributed, levels of attachment were around 50% lower than average. In other cases, the water film appeared to be even at one end of the tube, but not at the other. The effect, when looking down the tube, with bright illumination, was of a 'ring' of water which could occur at any point down the tube. These seemed to be related to a sudden sharp change in the number of attached cells, which could be observed microscopically. At one end of such tubes the cells were

Attachment levels after improving tube cleaning method

TABLE 2.1: Results of 22/4/85

Tube number	Average A ¹	Average B ¹	Overall Average	Overall Standard Deviation
1	42.0	41.8	41.9	29.6
2	40.6	34.3	37.4	30.3
3	39.3	37.4	38.3	20.4
4	35.9	45.9	40.9	37.2
5	34.7	41.8	38.2	42.9
6	34.5	39.9	37.2	32.7

TABLE 2.2: Results of 23/4/85

Tube number	Average A ¹	Average B ¹	Overall Average	Overall Standard Deviation
1	53.0	64.0	58.7	16.2
2	42.2	58.4	50.3	25.7
3	54.7	38.0	46.3	16.7
4	34.2	39.6	38.2	21.8
5	59.1	41.4	50.2	15.1
6	22.6	16.9	19.7	17.8

¹ The tubes were cut in half and opened out. Average A and Average B were the average numbers of cells attached in an area of 3125 μm^2 for each half (n=40). These are included to show the magnitude of variation in the readings.

Attachment levels for tubes cleaned using solutions of 1%
Lipsol, Micro and Teepol

TABLE 2.3: Results of 7/5/85

Deter- gent	Tube Number	Average A ¹	Average B ¹	Overall Average	Overall Std. Devn.	Remarks
Teepol	1	26.8	37.5	32.1	22.2	²
Lipsol	2	64.9	68.8	66.9	16.9	
	3	61.5	55.8	58.7	11.2	
	4	25.4	36.2	30.8	11.2	²
	5	55.3	59.9	57.6	12.0	
	6	79.0	52.5	65.8	21.6	³
	7	30.9	29.0	30.0	12.0	²
Micro	8	44.3	48.8	46.5	17.3	
	9	47.9	40.2	44.0	13.0	⁴
	10	56.9	54.6	55.8	27.1	
	11	56.6	59.4	58.0	14.6	³
	12	40.9	53.8	47.4	17.4	⁴
	13	55.2	25.0	40.1	18.7	³

¹ The tubes were cut in half and opened out. Average A and Average B were the average numbers of cells attached in an area of 3125 μm^2 for each half (n=40). They are included to show the magnitude of variation in the readings.

² These tubes had a patchy film of water when observed.

³ When these tubes were inspected, there appeared to be a 'ring', below or above which the water film was patchy. This appeared to affect the results considerably, as can be seen by comparing the difference between the averages for the two halves of the tube.

⁴ Although the water film appeared even, a slight difference was observed in attachment levels at one end of the tube.

sparsely distributed, whereas at the other side the level of attachment was much higher. A well defined junction separated the two areas. In three out of four cases when a 'ring' was observed in the water film, microscopic observation showed there was a sharp line at which the attachment level changed markedly at approximately the same position in the tube. In one case where no 'ring' in the water film was seen, there appeared to be a slight change in the attachment level at one point in the tube.

If the tubes which had been previously observed to have an uneven water film were disregarded, the average level of attachment for Lipsol cleaned tubes was 61.1 cells per $3125 \mu\text{m}^2$ (standard deviation 5.1, $n=3$, tubes 2, 3 and 5), 49.9 for Micro cleaned tubes (standard deviation 5.1, $n=3$, tubes 8, 10 and 12), and only 32.1 for Teepol cleaned tubes ($n=1$, tube 1).

This shows that attachment levels were considerably higher for tubes cleaned in Lipsol than in Micro, and the one tube tested which had been cleaned with teepol showed a lower level of attachment than either. The efficiency of the cleaning process still required improvement, if possible, as around half the tubes showed signs of not being perfectly clean when the water film on them was observed, and in one case in which the water film appeared even (though it was quite difficult to observe the film inside 7.5 cm lengths of 0.6 cm diameter tubing) the tube showed a jump in the attachment level at one end.

Thus it was decided to try increasing the concentration of Lipsol to 2%, and using some alternative detergents.

Attachment levels for tubes cleaned using solutions of 1% Lipsol, 2% Lipsol, 2% Tween 20, and 2% RBS 25.

TABLE 2.4: Results of 15/5/85

Deter- gent	Tube Number	Average A ¹	Average B ¹	Overall Average	Overall Std. Devn.	Remarks
Tween 20	1	52.5	42.5	47.5	-	²
RBS 25	2	72.4	75.4	73.8	-	
Lipsol 1%	3	79.1	72.8	75.9	16.8	
Lipsol 2%	4	80.4	92.1	86.2	23.7	
	5	87.3	66.9	77.1	21.7	
	6	78.4	86.1	82.3		
	7	101.0	73.5	87.3	30.7	
	8	94.9	81.3	81.3	16.7	²
	9	96.3	72.4	83.3	20.8	
	10	75.1	77.4	76.9	13.0	⁴
	11	100.5	70.5	85.5	25.9	²
	12	71.8	80.0	75.9	10.9	³
	13	84.0	93.8	88.9	31.5	³

¹ The tubes were cut in half and opened up. Average A and Average B were the average numbers of cells attached in an area of 3125 μm^2 for each half (n=40). They are included to show the variation of magnitude in the readings.

² These tubes had a patchy film of water when observed.

³ When these tubes were inspected, there appeared to be a 'ring', below or above which the water film was patchy. This appeared to affect the results considerably, as can be seen by comparing the difference between the averages for the two halves of the tube.

⁴ Although the water film appeared even, a slight difference was observed in attachment levels at one end of the tube.

Notes on the detergents used

TABLE 2.5.

DETERGENT	USE	REMARKS
Micro	Used at 2% for cleaning and decontamination.	Phosphate free, non-toxic, biodegradable.
Lipsol	Used for 'complete removal of all residues and radioactive contamination'. 2-5% for most applications.	Phosphate free, non-toxic, biodegradable, non corrosive.
Tween 20	Surfactant, used at 50 ppm.	Non-ionic surface active agent.
RBS 25	Cleaning and decontamination of laboratory glassware, surgical and pharmaceutical instruments etc. Replaces dichromate-sulphuric acid mixture. Diluted to 2% using demineralised water at 50°C. Rinse immediately after soaking.	Surface active agent.
Teepol	Completely removes grease films. If used in a hot concentrated solution, is a substitute for dichromate-sulphuric acid mixture for cleaning glassware.	Biodegradable, neutral, effective in hard and soft water, unaffected by acid solutions/chemicals containing salts of heavy metals. Relatively non-toxic.

The procedure for cleaning the tubes was kept the same, and the following detergents tested: 1% Lipsol, 2% Lipsol, 2% Tween 20 (Sigma Chemical Company Ltd., Poole), 2% RBS (Chemical Concentrates (RBS) Ltd., London).

The levels of attachment to tubes which had been cleaned using Lipsol detergent at a concentration of 2% were higher than for the tubes cleaned using other detergents (Table 2.4). The average number of cells per $125 \mu\text{m}^2$ was 83.1 for the Lipsol cleaned tubes, with a standard deviation 5.0 ($n=10$). This was a considerable improvement in the level of attachment and reproducibility of the counts. Similar results were seen in two repeat experiments. However, it was felt that the variability between the attachment levels was still too great: one of the causes was possibly the technique of counting the numbers of cells attached microscopically. High standard deviations were common, and increasing the number of areas counted appeared to have little effect on the standard deviation as a percentage of the mean. In addition the method became even more time consuming, taking several days to work out the results for one experiment. Hence it was necessary to develop an alternative technique of counting the numbers of cells attached, which would be more rapid, and allow the numbers of cells attached to be accurately determined (See Section 6.3, page 68). At the same time this was being done further information was obtained on the effect of using various acids to clean stainless steel. In experiments in which stainless steel capillary tubing was required to be cleaned to an extremely high standard, workers found that although dilute hydrochloric acid and dilute nitric acid gave poor results, 10% formic acid gave good repeatability.

The cleaning method was once again modified to try the effect of using formic acid. Stainless steel tubes (5 cm sections) were degreased by soaking in chloroform for 1 hour. They were then rinsed three times in 10% formic acid, and soaked for one hour in 10% formic acid. Then they were rinsed 3 times in 3% Lipsol detergent, placed in a beaker filled with 3% Lipsol detergent, and sonicated for 5 minutes. The tubes were rinsed 10 times in distilled water and sonicated in distilled water for 5 minutes. They were rinsed 10 times in distilled water and left to soak in distilled water for 24 hours. After this they were rinsed 10 more times in distilled water, left to dry, and then wrapped in individual stainless steel parcels and sterilised in an oven at 160°C for 2 hours.

This experiment differed slightly from the previous ones in that the cells were labelled with tritiated leucine (see Section 6.3.1, page 71, for details of labelling method). Apart from this, the procedure was identical. The results were converted into numbers of cells per 3125 μm^2 to enable comparison with the results shown in Tables 2.1 to 2.4. The results are summarised in Table 2.6.

It can be seen that the average numbers attached are very similar, but the tubes cleaned using formic acid showed much less variability in numbers attached. Hence all tubes used in subsequent experiments were cleaned using the method which incorporated formic acid.

6.3 Counts of attachment level using a radiolabelling technique

As previously mentioned, increasing the area counted when using the epifluorescent microscope, did not improve the

The effect of using formic acid in the cleaning procedure

TABLE 2.6: Results of 7/8/85

Tube cleaning method	Average dpm ¹	Standard deviation (as % of average)	Number of tubes	Average no. cells per 3125µm ²
No formic acid	32011	18.9%	9	62.7
With formic acid	32232	8.1%	9	63.1

¹ Disintegrations per minute. The number of cells attached were directly related to dpm (see section 6.3 for details of the labelling technique).

² The results were converted into numbers of cells per 3125 µm to enable comparison with Tables 2.1 to 2.4. The number of cells per dpm was 885.4, found by measuring the counts per minute of a 100 µl sample which had a cell density of 1.25×10^8 cells/ml. Total inside surface area of tube (7.5 cm long tube) was 14.14 cm².

standard deviation in many cases. This was probably due to the patchy nature of the attached cells, and it would have required a prohibitively long time to count a sufficiently large area to significantly reduce the standard deviation of the counts.

It was decided to try to measure the numbers of cells attached to the inside of a tube by prelabelling the organisms using a radiotracer and measuring attachment by determination of surface-bound radioactivity. Pre-labelling of cells with tritiated leucine before the attachment stage had several advantages. It meant that the numbers of cells attached could be determined for the entire tube, thus eliminating the problems caused by variations in the distribution of the cells over the surface on a microscopic scale. In addition, it was a relatively quick and simple method, and as it was foreseen that radiotracers would be used in subsequent uptake studies, it was felt that the method would save time as two distinct tracers such as ^3H and ^{14}C could be counted simultaneously.

The main difficulty in setting up the method was to establish a suitable technique for measuring numbers of cells attached to the inside of 5 cm stainless steel tubes: it was possible to cut up the tube and put it straight into scintillant, but the method would have been time consuming and there was certain to be quenching due to the presence of the stainless steel surface in the vial of scintillant; the method which was ultimately adopted involved removing the cells from the surface first, using tissue solubiliser.

6.3.1 Pre-labelling procedure

Cells which had been grown up for 24 hours in glucose medium were harvested by centrifugation (15 min; 10,000 g_{av}; 10 mins) and resuspended in 20 ml 0.04 M KH₂PO₄ buffer (adjusted to pH 7.4 using 1 M NaOH); 20 µCi (20 µl) of L-[4,5-³H] Leucine (1 mCi/ml, 45 Ci/mmol) were added, and the cells were then incubated at 15°C on a shaking incubator for 1 hr. The ³H-labelled cells were washed 3 times (10 min; 10,000 g_{av}; 15°C) in 20 ml KH₂PO₄ buffer. The labelled cells were then resuspended in glucose medium to an optical density of 0.9 at 540 nm in a colorimeter (Corning Corimeter 252) and used in the attachment incubation stage. The extent of leakage from the labelled cells was determined by counting 100 µl duplicate quantities of the final wash supernatant: in all cases the level of leakage was less than 1%. (Leucine leakage from the cells was negligible for a period of at least 12 hours.) To estimate the amount of radioactivity taken up per organism, a sample of the labelled cell suspension was diluted 1:50 in sterile distilled water, and the number of cells counted using a counting chamber (Weber Scientific Int. Ltd., Lancing, Sussex). A 100 µl aliquot was counted in the scintillation counter. The dpm (disintegrations per minute) per cell could then be calculated.

Cells which were attached to tube surfaces were eluted using Lumasolve tissue solubiliser (LKB Instruments Ltd., Milton Keynes). The Lumasolve was diluted 5:1 with distilled water, which increased the efficiency of elution. The 5 cm stainless steel tubes were placed in 7.5 cm test tubes, and 2.3 ml tissue solubiliser was added, which just covered the stainless steel tube. The test tubes were stoppered with 6 mm

rubber bungs, having carefully wiped the inside surface of the test tubes several times with absorbent tissue, to remove any traces of tissue solubiliser, which removed the dye from the bungs, and caused quenching when the sample was counted in the scintillation counter. The test tubes were then placed in an oven at 50°C overnight (approximately 16 hours incubation). The contents of each tube were then decanted into an empty glass scintillation vial. The remaining traces of tissue solubiliser were removed by rinsing the tube 4 times with a 2.5 ml aliquot of Lipoluma scintillant (LKB Instruments Ltd., Milton Keynes), each portion being decanted into the vial containing the tissue solubiliser. The vial was whirlmixed briefly, and the outside wiped with a tissue before it was placed in the scintillation counter.

Liquid samples were added to glass vials containing 10 ml Lipoluma and 2.3 ml Lumasolve (i.e. the same amounts of scintillant and tissue solubiliser as for attached cells). The vial was then mixed, and wiped with a tissue, before being placed in the scintillation counter.

The samples were counted in a liquid scintillation counter (System LS-7000, Beckman Instruments Inc, Fullerton, Calif.). The extent of quenching was established using the H number system; an external standard monitoring system based on the Compton electron spectrum generated by a ^{137}Cs gamma source (Horrocks, 1977).

It was important that the scintillant used was as efficient as possible, as in some experiments tubes would have very few attached cells: for example, in the case of a detachment experiment in which high flow rates were used.

In the first trials two different scintillants were

used. The first was a scintillation cocktail consisting of 2:1 (v/v) toluene; Triton X-100, and 0.6% (w/v) of the scintillator 2-(4'-tert-butylphenyl)-5-(4"-biphenyl-1-3',4,-oxadiazole) (butyl PBD). The second was a commercial cocktail, Lipoluma, which was compatible with the Lumasolve tissue solubiliser which was used to elute the cells from the surfaces. It was found that the Lipoluma scintillant was approximately twice as efficient as the toluene based cocktail, and this had the additional advantage of being compatible with the tissue solubiliser. This scintillant was therefore chosen.

6.3.2 Note on the scintillants used

At the start of this study 'Lumasolve' tissue solubiliser was used, which was compatible with 'Lipoluma' scintillant. Fresh stocks of these could not be obtained as they were no longer available. Optisolve tissue solubiliser was therefore used as a replacement, with OptiScint 'Safe' scintillant. The two tissue solubilisers were compared, and the results obtained were very similar. Subsequently, it was noticed that there was some chemiluminescence if the OptiScint scintillant was used for aqueous samples, though this did not occur with the tube samples which contained little water. Optiphase 'MP' was used for aqueous samples after this, but the OptiScint was still used for tube samples as tests showed that it was more efficient.

7. Measurement of uptake of glucose

The glucose taken up by the attached cells was measured by adding a small quantity of labelled glucose to the glucose medium which was used to fill the rig. In most experiments an

aliquot of 20 $\mu\text{Ci/l}$ ^{14}C glucose was added just before the start of an experimental run.

In order to calculate the amount of glucose taken up per cell, the cells were eluted from the tubes after the run as described on page 71, and the vials were counted using a programme designed for dual labelled samples (Programme 7; Beckman LS-7000 scintillation counter). The actual number of disintegrations per minute in each channel was calculated using a method described by Horrocks (Beckman Biomedical Technical report 1043-NUC-76-56T). A computer programme was written to enable this reiterative calculation to be performed rapidly. It was necessary to establish the counting efficiency of each radiolabel in both of the counting channels (ie in the ^3H channel and the ^{14}C channel), and the relationship between counting efficiency and the degree of quenching in each selected counting channel. The level of quenching of each sample was determined by the H number system, an external standard monitoring system (Horrocks, 1977).

8. Calculation of glucose uptake

The various stages of the calculation of glucose uptake per cell are detailed below:

A. Calculation of dpm per tube for ^3H leucine and ^{14}C glucose:-

The actual number of disintegrations per minute (dpm) were calculated from the counts per minute (cpm) taken from the scintillation counter print-out using a simple computer programme.

Listing of programme:

```

10  CLEARW 2:FULLW 2:REM CLEARS OUTPUT WINDOW
20  PRINT "INPUT E12,E21,E22,B1,B2"
30  INPUT E12,E21,E22,B1,B2
40  LPRINT "Ch1 cpm","Ch2 cpm","E11","ch1 dpm",ch2 dpm"
50  LPRINT
60  PRINT "PLEASE ENTER DATA"
70  INPUT C1,C2,E11
80  C1=C1-B1 : C2=C2-B2 : REM TO SUBTRACT BACKGROUND READING
90  D2=C2/E22 : REM DIVIDES COUNTS IN 14C CHANNEL BY 14C
    COUNTING EFFICIENCY FOR THAT CHANNEL
100 FOR N=1 TO 10 : REM SIMPLE REITERATIVE CALCULATION
110 D1=(C1-(D2*E21))/E11
120 D2=(C2-(D1*E12))/0.8
130 NEXT N
140 PRINT
150 D1=INT(D1+0.5) : D2=INT(D2+0.5) : REM CONVERT TO INTEGERS
160 LPRINT C1,C2,E11,D1,D2
170 PRINT
180 GOTO 50

```

Key to variables:-

E11 Efficiency of isotope 1 (³H) in channel 1
 E12 Efficiency of isotope 1 (³H) in channel 2
 E22 Efficiency of isotope 2 (¹⁴C) in channel 2
 E22 Efficiency of isotope 2 (¹⁴C) in channel 1
 C1,C2 Counts per minute recorded in channel 1 and 2,
 respectively
 B1,B2 Background counts recorded in channels 1 and 2,
 respectively
 D1,D2 Calculated disintegrations per minute in channel 1 and
 2, respectively

The efficiencies were determined from a quench curve which showed plots of the efficiency of the two isotopes in the two channels versus H number. E11 was the only value which varied significantly with H number, and this was read from the graph for each result.

B. Extract from a typical print out:-

Ch1 cpm	Ch2 cpm	E11	ch1 dpm	ch2 dpm
3001	569	.355	8168	507
3564	777	.355	9628	731
4566	989	.355	12339	928

C. Calculation of number of cells per tube:-

Duplicate 50 μ l samples of a suspension containing ^3H -leucine labelled cells were counted in the scintillation counter. The number of cells per ml in the suspension were checked by (a) counting using a counting chamber; (b) checking the absorbance at 540 nm (SP1700 spectrophotometer). The dpm/cell could therefore be calculated.

Example:

Optical density (OD) of sample: 0.723 (sample diluted by 5 in order to read OD)

Average dpm (^3H) of 50 μ l sample: 22781 (sd=123, n=2)

Cells/ml = 1.23×10^7 (from graph of OD versus cell numbers)

Therefore:
$$\text{Dpm/cell} = \frac{22781 \times (1000/50)}{(1.23 \times 10^7) \times 5}$$

Using the first line of data in the example printout given above, the number of dpm in channel 1 (^3H) was 8168. The number of cells per tube equals dpm in channel 1 multiplied by (1/dpm per cell).

$$\begin{aligned} \text{ie Cells per tube} &= 8168 \times 13498 \\ &= 1.10 \times 10^8 \end{aligned}$$

D. Uptake per cell:-

In most cases 20 $\mu\text{Ci/l}$ ^{14}C glucose was used in the rig medium. In the example chosen 22 μCi ^{14}C glucose was added to 1.1 l medium which contained 0.2% (w/v) unlabelled glucose (2.0 g/l). It was necessary to calculate the number of dpm ^{14}C glucose present per g of unlabelled glucose:

$$\text{Dpm/g unlabelled glucose} = \frac{2.2 \times 10^4 \times 22}{2.2} = 2.2 \times 10^7$$

To calculate the total amount of glucose taken up per tube, the dpm in channel 2 (taken from the first line of the typical print out shown above in section C) was divided by the dpm/g unlabelled glucose.

$$\begin{aligned}\text{Total glucose taken up per tube} &= \frac{507}{2.2 \times 10^7} \\ &= 2.30 \times 10^{-5}\end{aligned}$$

Assuming that no cell division occurs,

$$\begin{aligned}\text{uptake per cell} &= \frac{\text{Total glucose taken up per tube}}{\text{Total number of cells per tube}} \\ &= \frac{2.30 \times 10^{-5}}{1.10 \times 10^8} \\ &= 2.09 \times 10^{-13} \text{ g/cell}\end{aligned}$$

Table 8.1 (page 191) shows the results of all uptake experiments in terms of uptake per cell.

9. Improvement of the 'attachment incubation' procedure

Cells were attached to the stainless steel tubes using the method described on pages 50 and 56. However, it became clear that this technique had several drawbacks. The major problem was due to the fact that not all bungs were identical in size (this problem was increased after the bungs had been sterilised several times), and this affected the area available for attachment. In addition, it was difficult to ensure that the tubes all rolled evenly in the shaking incubator, and on occasions tubes became stuck, which could result in uneven attachment.

A new method for attaching the tubes was developed which ensured that conditions were identical for all tubes. The stainless steel tubes were linked together with 2 cm sections of silicone tubing (i.d. 6 mm). At either end of the chain of tubes, a longer section of silicone tubing linked to a

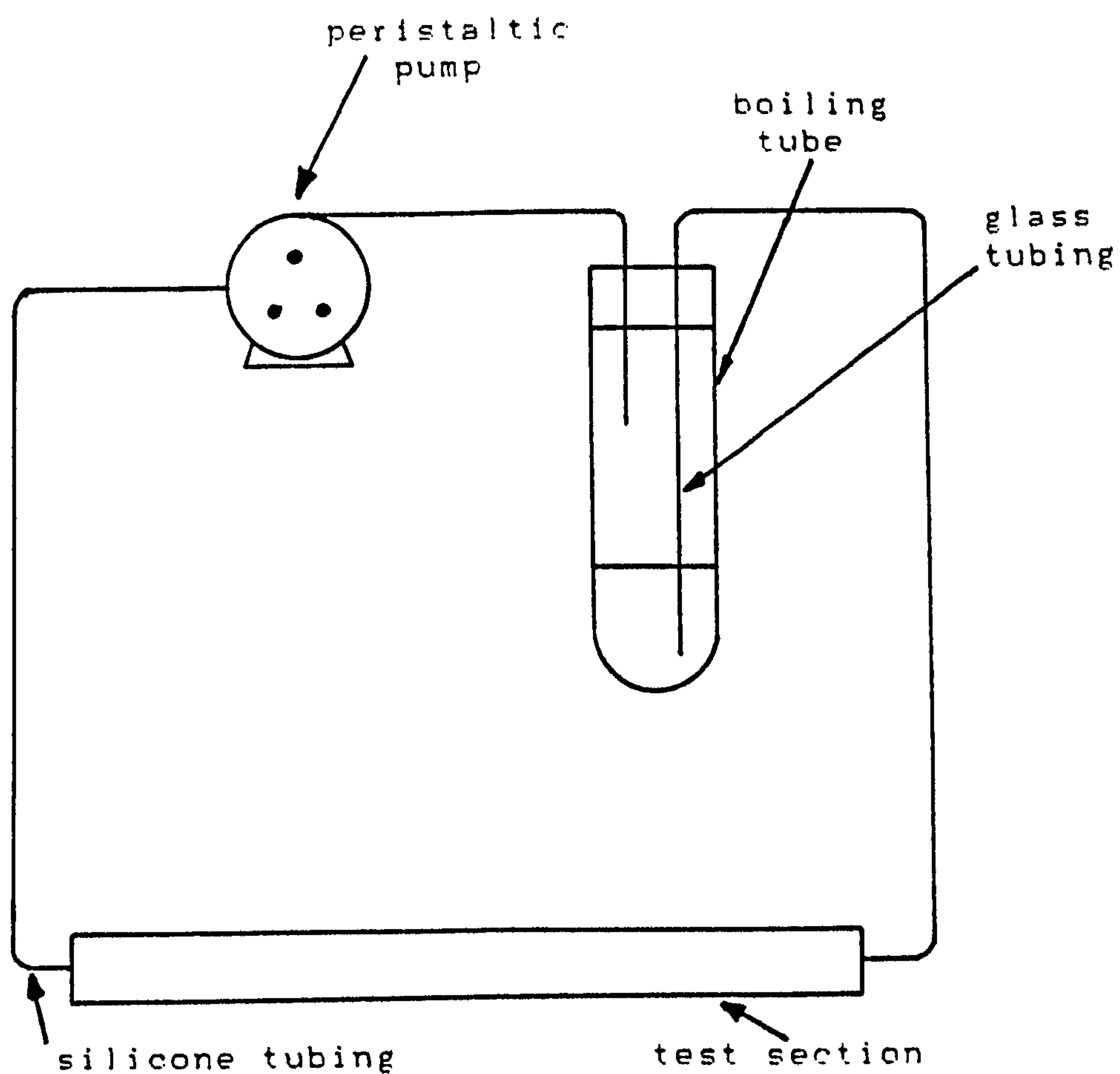
boiling tube (see Fig 2.14). The apparatus was sterilised and a culture of cells could be pumped around at 0.1 m/s (0.17 l/min) using a peristaltic pump. Cells were grown up, labelled and resuspended as described previously (pages 50 and 56). The culture was transferred aseptically into the boiling tube, and then pumped around the circuit for a period of two hours; this was the same period as was used in the previous attachment method. Precautions were taken to ensure that there were no air pockets in the circuit.

The number of cells attaching to each tube during the two hour period was slightly less when this 'flow system' was used to attach the cells: however the average difference was less than 10%. The change in attachment method did, however affect the numbers of cells which detached in the flow rig (see Chapter 3, page 80).

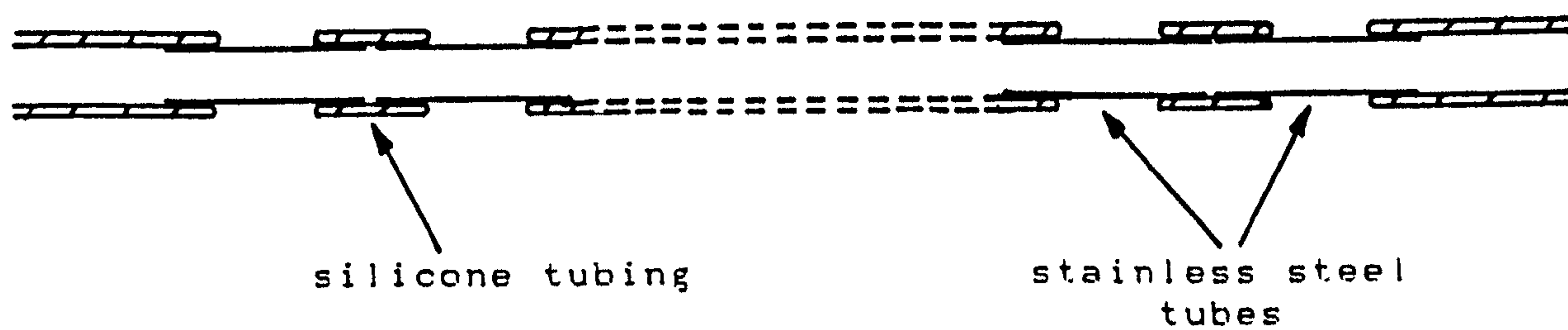
10. Chemicals

Reagents were obtained from Fisons or British Drug Houses (BDH), unless specified otherwise in the text, apart from acridine orange and HEPES buffer which were obtained from Sigma. All radiochemicals were supplied by the Radiochemical Centre, Amersham.

2.14 Diagram of the flow system for attaching
cells to the stainless steel tubes.



DETAIL OF TEST SECTION:



CHAPTER THREE

ANALYSIS AND CHARACTERISTICS OF BIOFILM

1. Detachment Experiments

1.1 Introduction

The aims of the initial experiments using the experimental flow rigs were to measure the level of detachment of cells at different flow rates and to determine whether there was a 'critical' flow rate, at which the detachment level increased markedly. In addition, the change in the level of detachment with time was studied.

The stainless steel tubes, with a pre-attached monolayer of cells, were placed in the flow rig, as described on pages 51 and 77. The attached cells were then exposed to various flow rates over 0.5 or 1.0 hour run periods. After this the numbers of cells attached per tube were determined. The numbers of cells which were still attached were compared with the average number of cells attached prior to placing the tubes in the flow rig (the latter found by using another set of tubes, and averaging the number of cells attached following the attachment incubation). From this the percentage of cells which had detached were calculated as described on page 53. In the earlier experiments the cells attached were determined microscopically, whereas in all other experiments, the numbers were found using the ^3H labelling technique.

1.2 Results and discussion

The variation in detachment levels from experiment to experiment was quite considerable and obviously small variations in experimental conditions can have a marked effect. It

is difficult to draw firm conclusions about the factors which affect detachment levels, because in any given experiment, the level of detachment was similar for each tube, but on some occasions the detachment levels measured in two identical experiments (carried out at different times) showed considerable variation.

Before the leucine labelling technique was used to determine the numbers of cells attached, there was a greater variation between individual tubes. It was noticeable that following the change of attachment method on 4/2/86, the detachment levels decreased considerably to levels which were generally below 20%, and often much less. This is likely to be due to the fact that cells attached using the flow system (after 4/2/86) were exposed to a low shear stress during the attachment stage.

1.2.1 Effect of flow rate on detachment

Fig. 3.1 shows the effect of flow rate on the numbers of cells detaching/remaining attached. It can be seen that the percentage detachment was lower at all flow rates for films which were attached in the flow system (from results obtained after 4/2/86, when this method was implemented). The plot of the percentage of cells remaining attached versus flow rate enables the results to be compared with similar plots obtained by Duddridge et al (1982), which show the effect of surface shear stress on the attachment/detachment of Pseudomonas fluorescens using stainless steel surfaces. This study shows that, although individual results vary, there is a significant steep drop in the numbers of cells remaining attached at surface shear stresses of 10 to 12 N/m², corresponding to flow rates of 2.70 to 3.3 l/min. The data

3.1 The effect of flow rate on detachment of cells
at a glucose concentration of 2.0 g/l.

Run period: 0.5 hour.

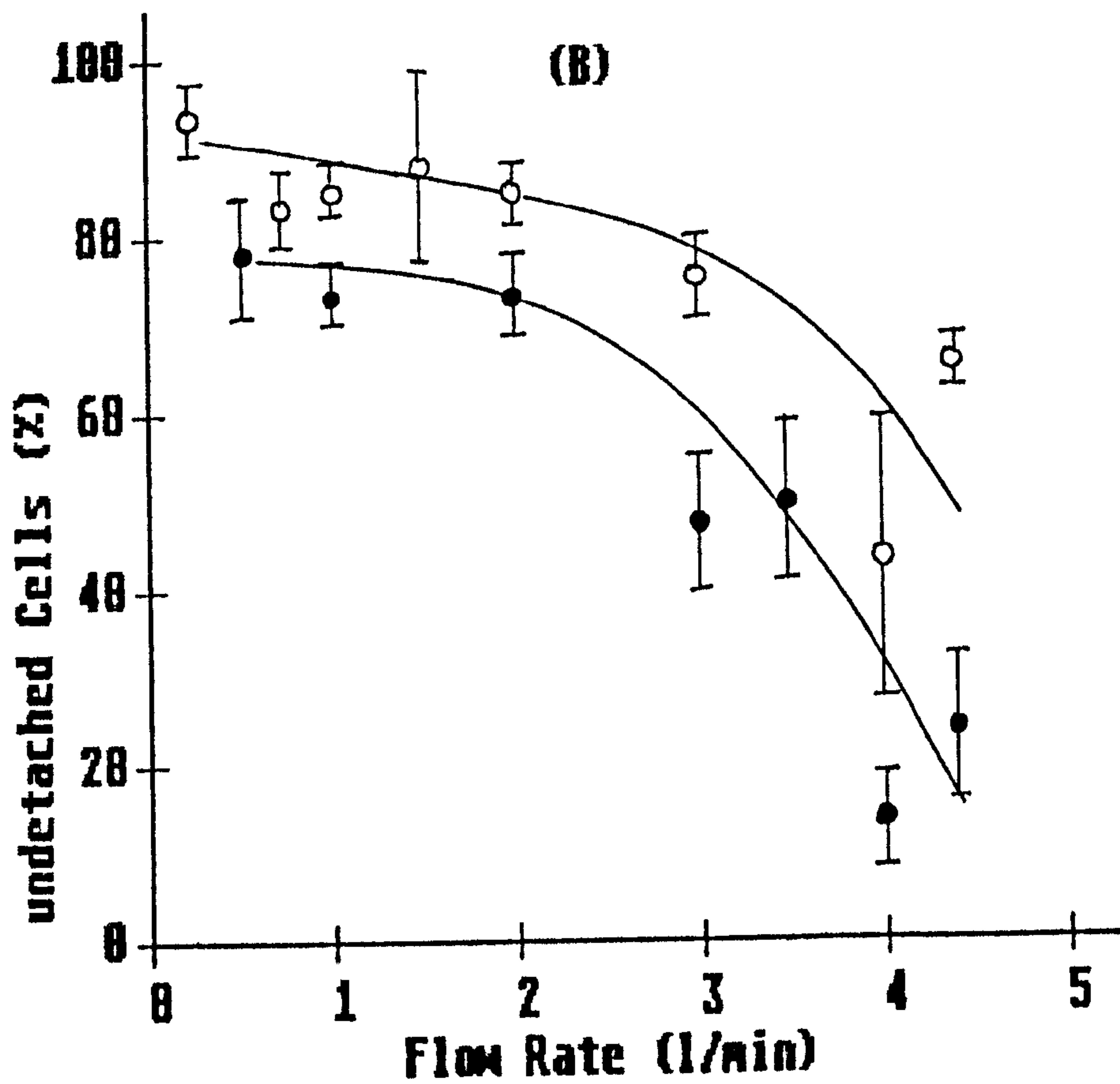
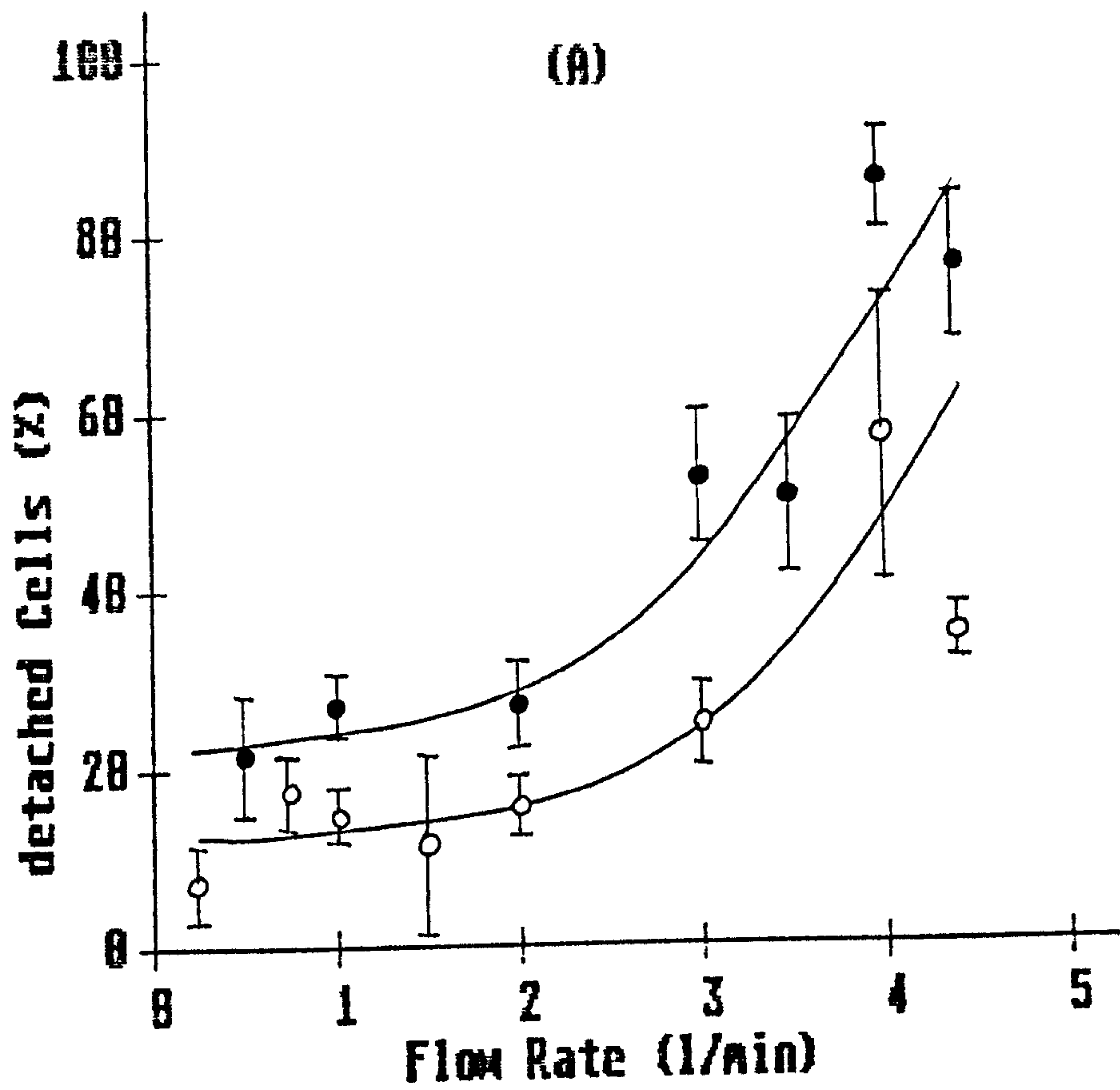
A: numbers of detached cells versus flow rate.

B: numbers of cells remaining attached versus
flow rate.

Key:

- Averaged results of experiments carried out
prior to 4/2/86
- Averaged results of experiments carried out
after 4/2/86

NB. The method of attaching cells to the tubes was
changed on 4/2/86 - see Chapter 2.



from the present study is not as detailed as these results, but the graphs appear to follow similar patterns. However, the drop in numbers of cells remaining attached at flow rates above 2.7 l/min is more gradual than that shown by Duddridge's study. This could be partly because the number of flow rates at which results were obtained were fewer in this study, and the results plotted show the average results for several experiments. There was no 'critical' flow rate at which a dramatic drop in the numbers attached occurred, but a fall in numbers attached occurred at approximately 3 l/min, similar to the value found in Duddridge's study.

1.2.2 The change in detachment with time

Several experiments were carried out in which the run length was varied: ie tubes were removed from the rig at intervals (see Fig 3.2). In all of these experiments a flow rate of 2 l/min was used. It was not possible to measure detachment for periods of less than five minutes. After five minutes, the level of detachment appears to be low as the number of cells per tube remains approximately constant. (Detachment may still be occurring but it must be roughly matched by the level of reattachment.) Hence it would appear that a large percentage of the cells detach within the first five minutes, and after this, the cells which remain are relatively firmly attached.

1.2.3 The effect of glucose concentration on detachment

The glucose concentration in the rig does not appear to significantly affect the level of detachment except possibly at very low glucose concentrations (Fig 3.3). In order to draw firm conclusions, more experiments, using a wider range of glucose concentrations, would be necessary.

3.2 The effect of time on detachment of cells at a
 flow rate of 2 l/min.

A: 0.2 g/l glucose.

 Results of 19/2/86.

B: 0.5 g/l glucose.

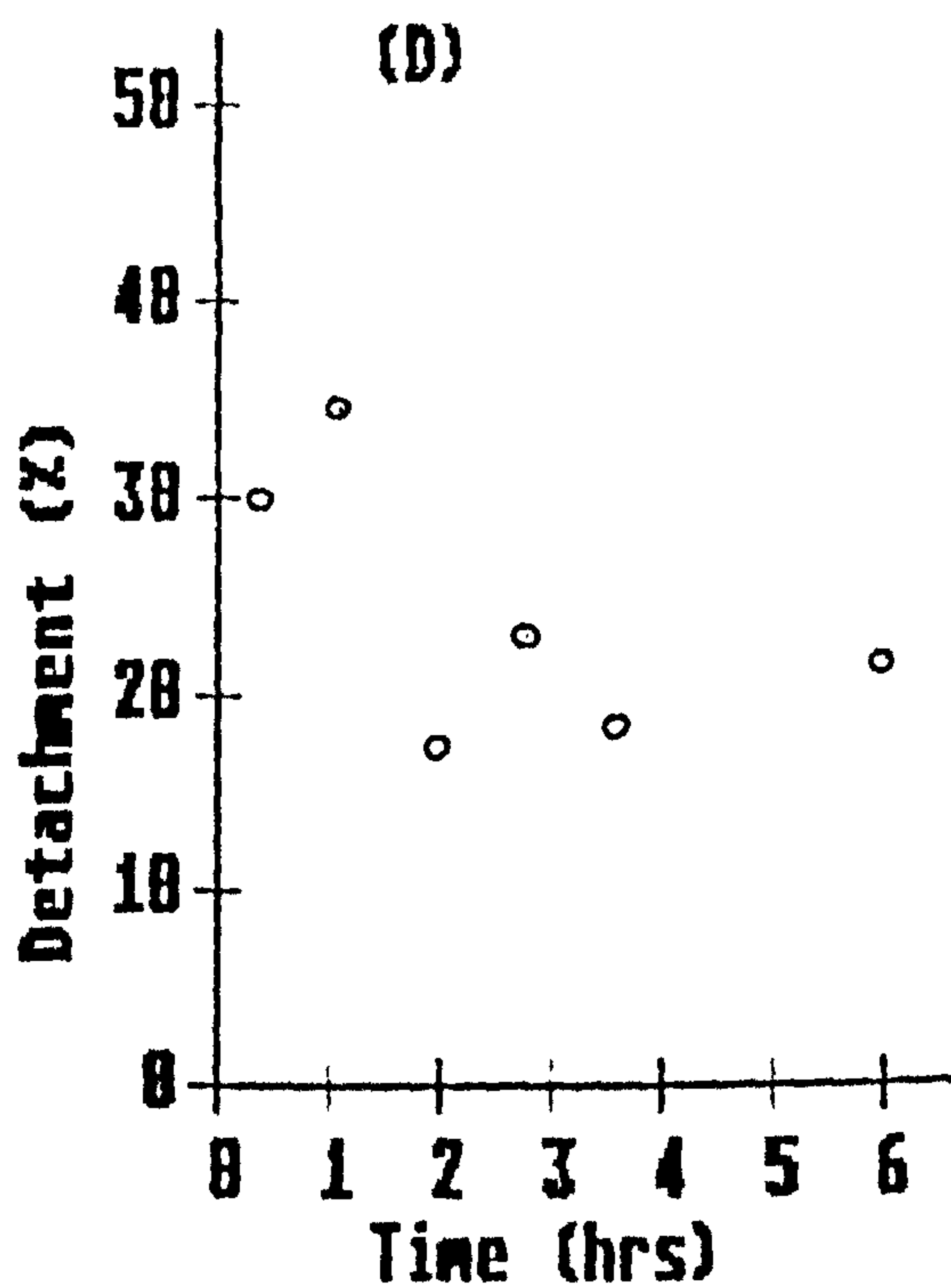
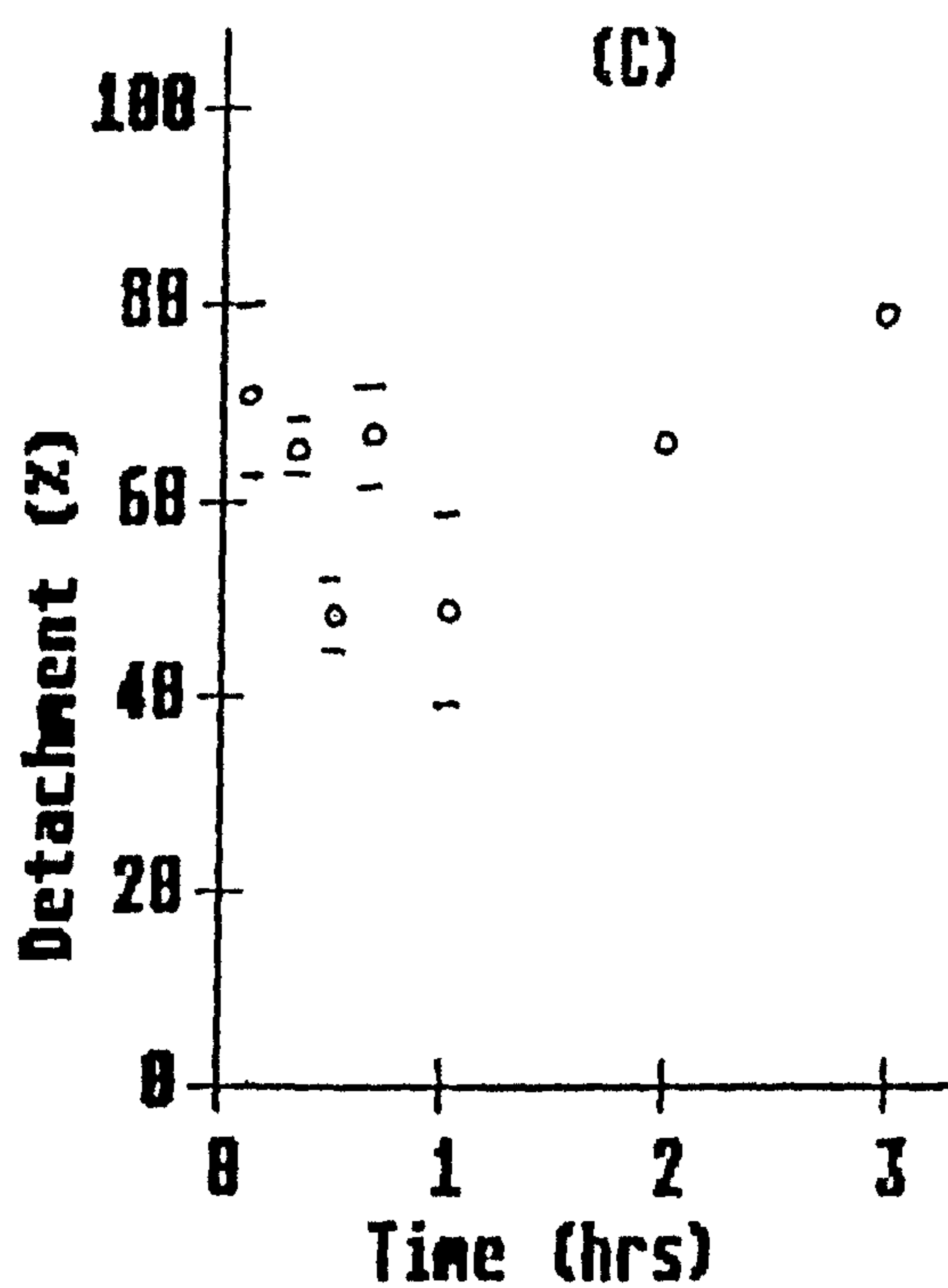
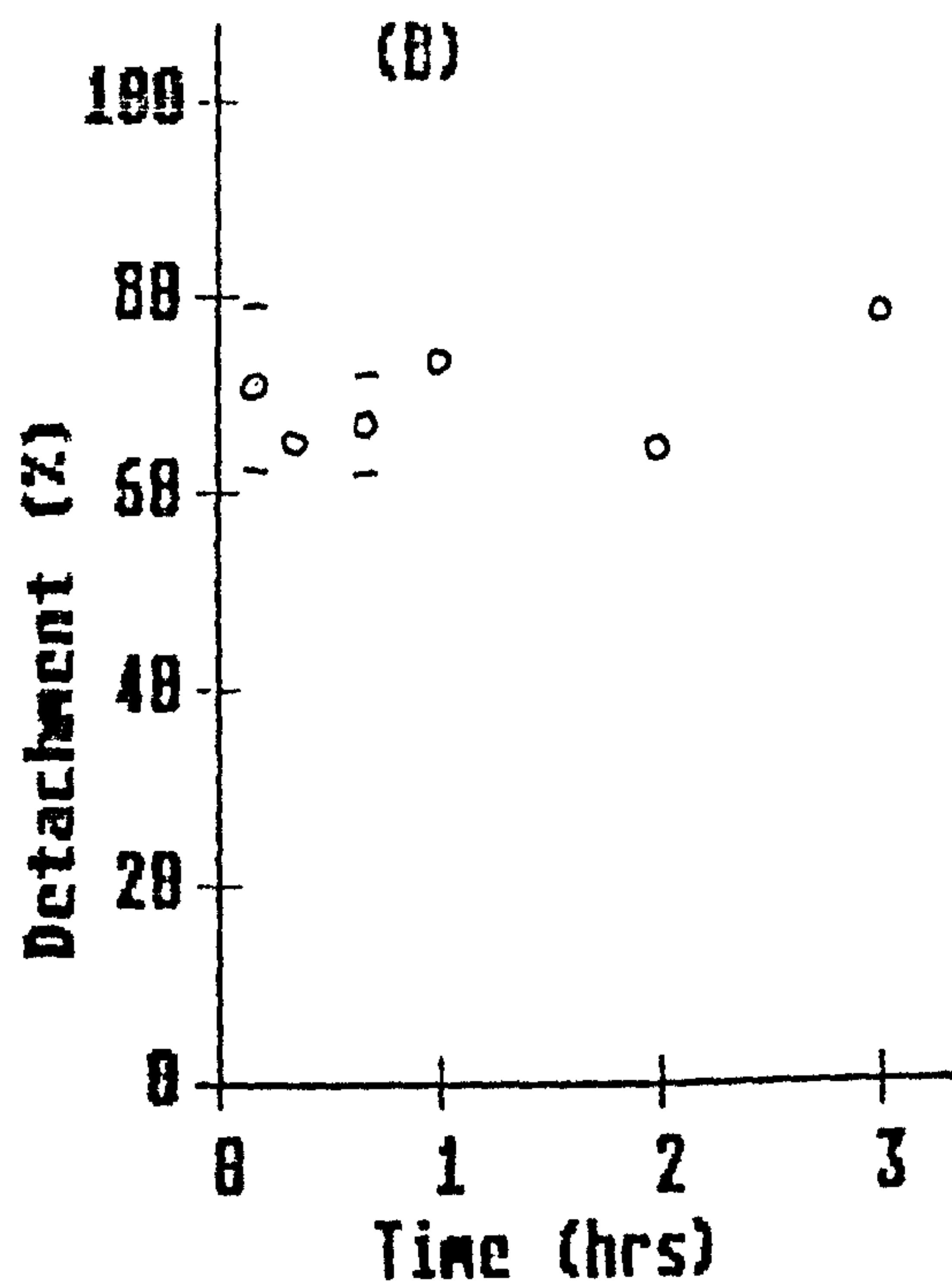
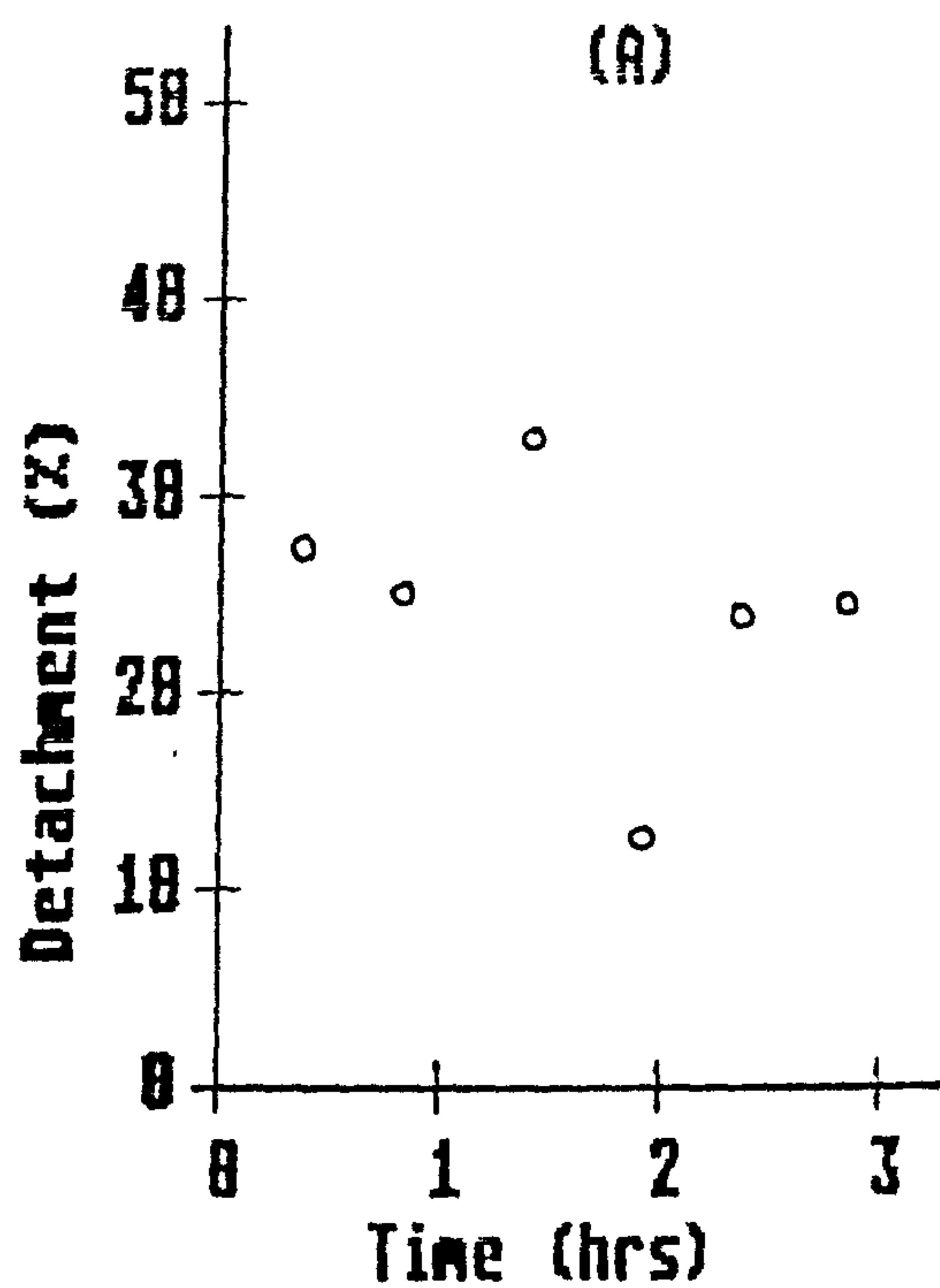
 Average of results from 10/10/85, 16/10/85 and
 24/10/85.

C: 0.5 g/l glucose.

 Average of all results.

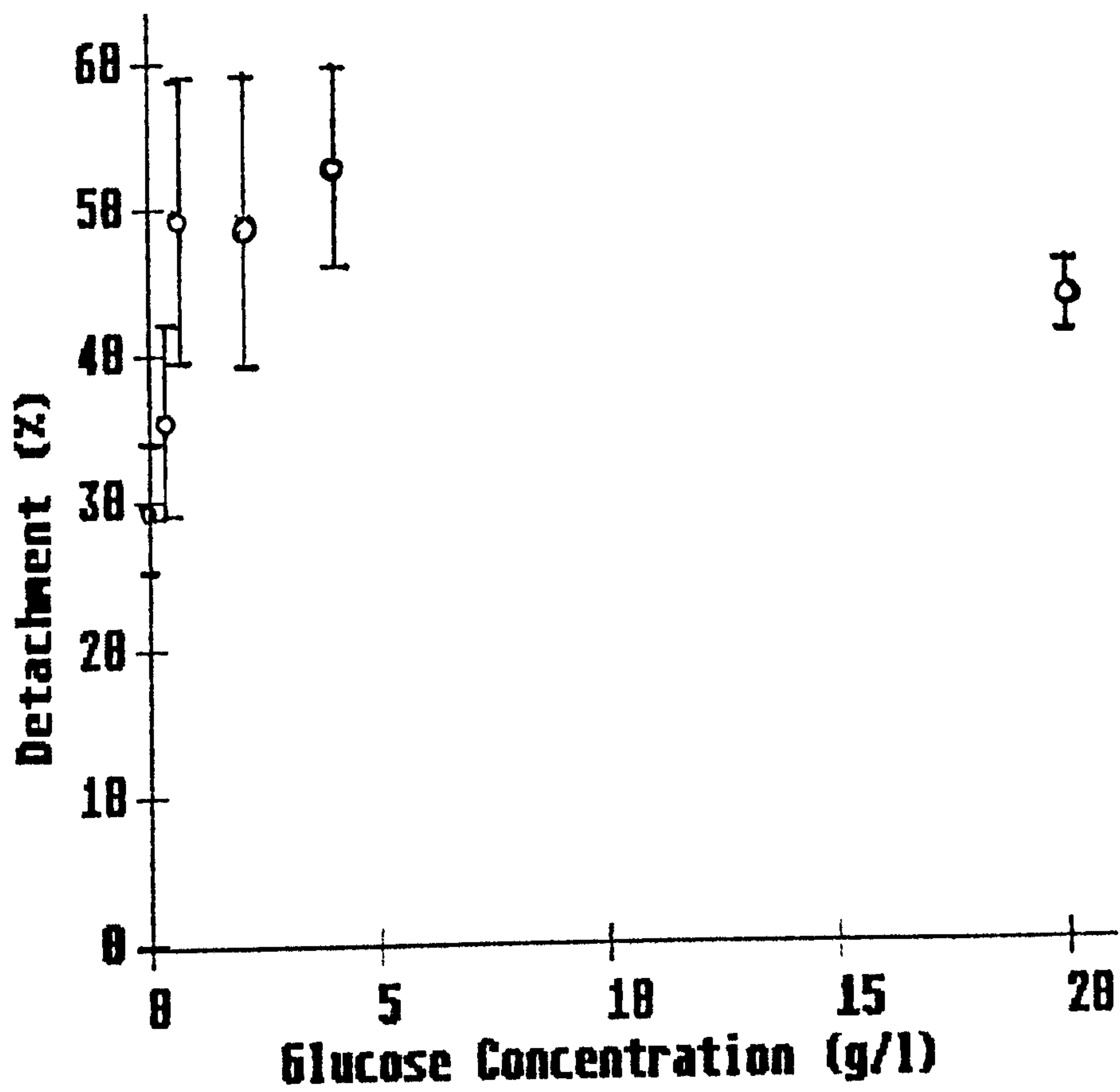
D: 2.0 g/l glucose.

 Results of 29/1/86.



3.3 The effect of glucose concentration on detachment of cells.

2 l/min flow rate:



2. The effect of increasing experimental run length

2.1 Introduction

A series of experiments were carried out at a fixed flow rate of 2 l/min in order to characterise the changes in uptake of the biofilm. Tubes were removed from the rig after different time periods; the glucose concentrations used in the flow rig in these experiments were 0.2, 0.5 and 2.0 g/l.

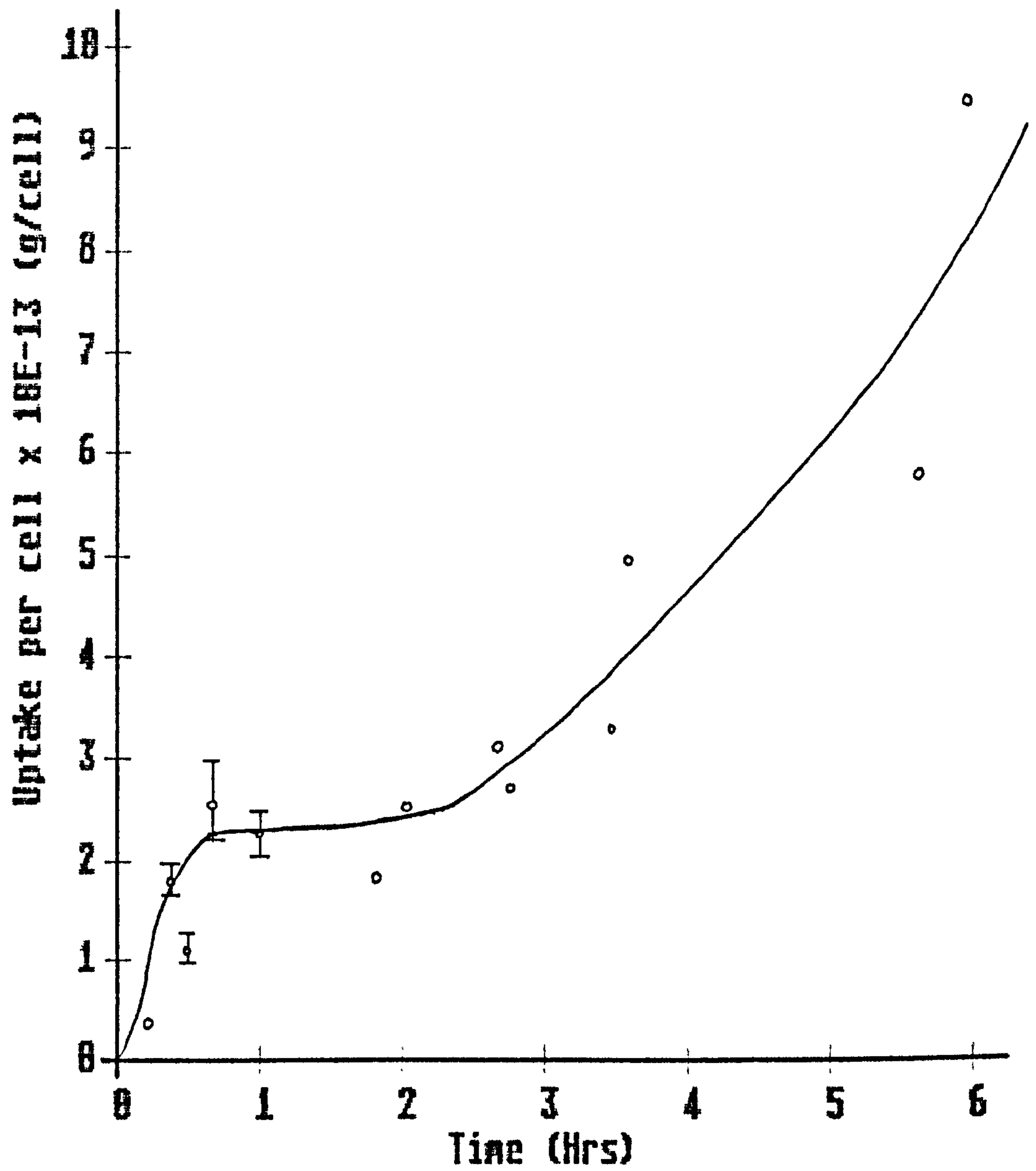
2.2 Results and discussion

The results for these experiments are shown in Figs 3.4, 3.5, and 3.6. It can be seen that the results obtained at each glucose concentration follow approximately the same pattern. Initially, during the first 30 to 60 minutes, there is a rapid accumulation of substrate; this is followed by a period of around one hour when there is little further increase in the uptake of substrate, after which an exponential increase appears to take place. The growth of the biofilm must be exponential during this period, in order to cause this increase in uptake. Although the number of cells attached to the tubes at the beginning of each experiment, and also the numbers of those cells which did not detach from the tube surface during a run, could be determined by measurement of the leucine label, the growth of cells during a run could not be determined.

These graphs emphasise the difficulty of choosing a suitable run period. If the run period is less than 0.5 hour, the uptake of substrate is linear. Following this, the uptake rate decreases, and after two to three hours the uptake is exponential due to the growth of the attached cells. The length of each of these phases varies depending on the

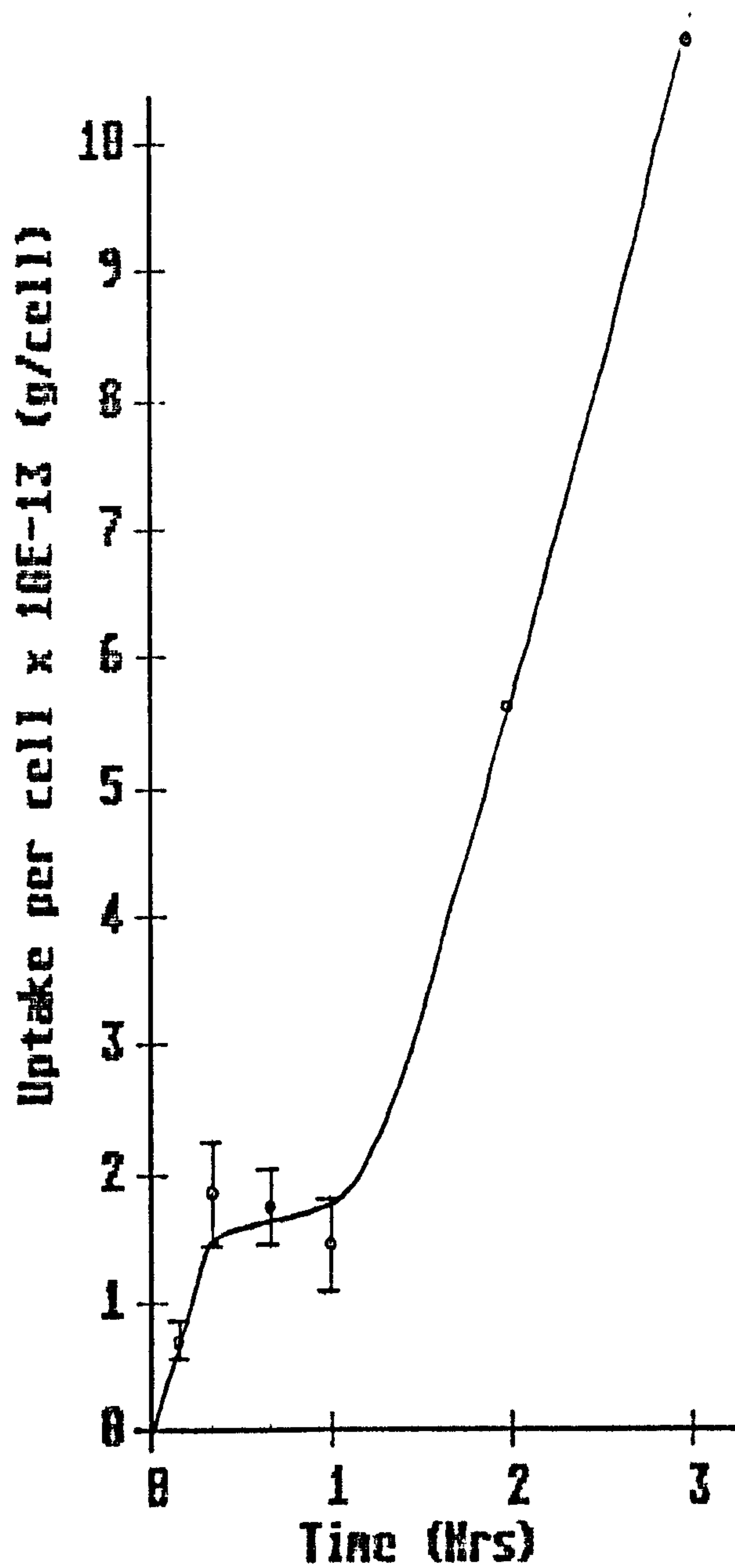
3.4 Uptake versus time for attached cells exposed to a flow rate of 2 l/min and glucose concentration of 2.0 g/l.

Note: Where no error bar is shown, the point is derived from a single result.



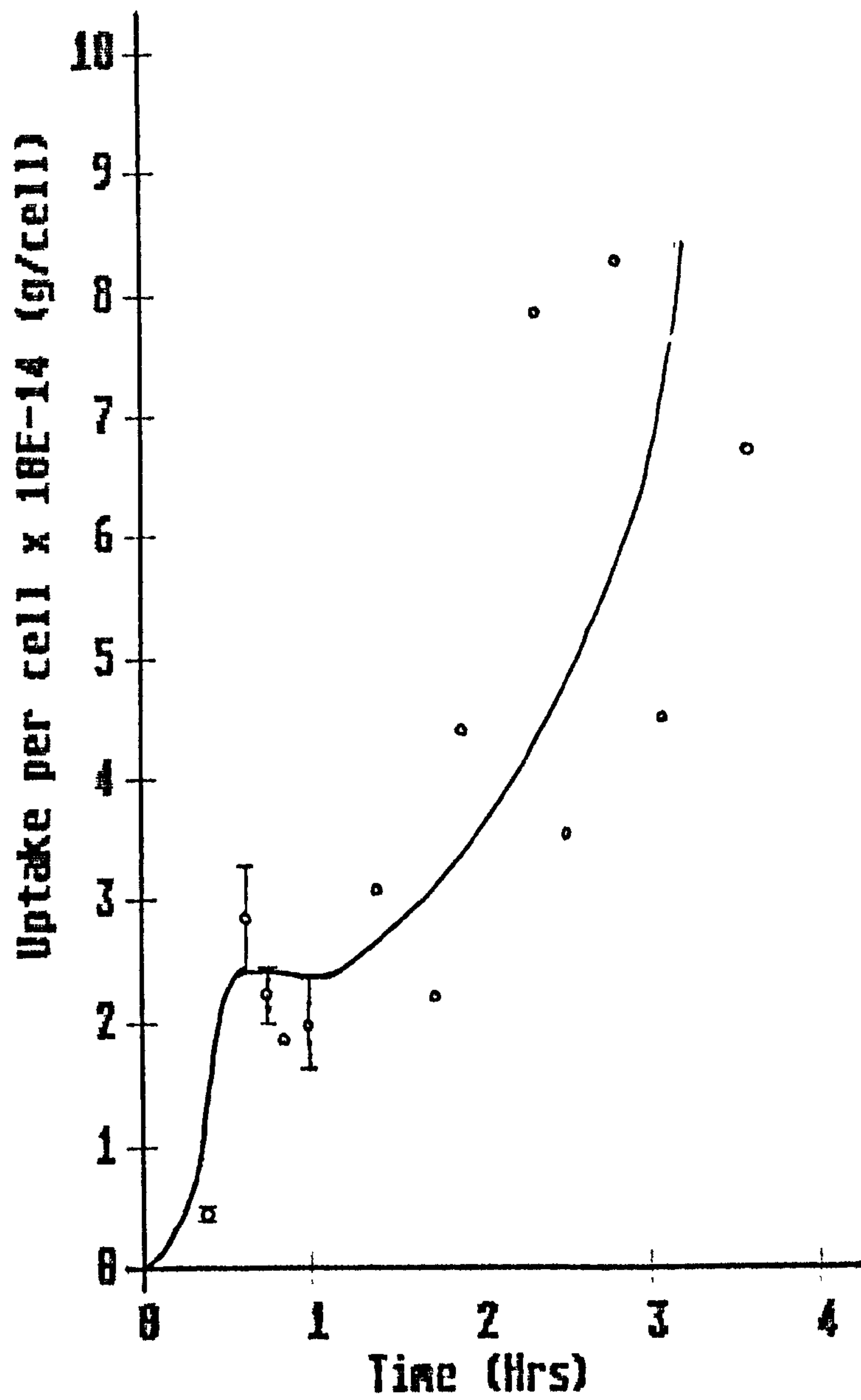
3.5 Uptake versus time for attached cells exposed to
 a flow rate of 2 l/min and glucose concentration
 of 0.5 g/l.

Note: Where no error bar is shown, the point is derived
 from a single result.



3.6 Uptake versus time for attached cells exposed to a flow rate of 2 l/min and glucose concentration of 0.2 g/l.

Note: Where no error bar is shown, the point is derived from a single result.



substrate concentration. For longer run periods, a method of measuring the number of cells on the tube surface at the end of the run was required. Such a method would also be useful for short run periods, in order to check the validity of the assumption that no growth occurred during a run. A considerable time was spent in the attempt to establish such a measure (see pages 92-124).

During the initial period of up to approximately 30 to 60 minutes, and the period of rapid increase which occurred after about 2 hours, rates of uptake could not be accurately determined. The approximate rate of uptake at each glucose concentration was proportional to the amount of glucose present except at 0.5 g/l glucose (see Table 3.1).

Many of the experiments to date in which biofilms have been studied under flowing conditions have been lengthy (often lasting weeks or months); during this time biofilms may develop to a considerable thickness. It was not possible to carry out experiments with such long run periods in this study due to the small scale of the flow rig.

In some previous studies, substrate uptake by biofilms exposed to flowing conditions has been investigated. However, in most cases this has been limited to substrate uptake by thick biofilms which have been established for considerable periods (Trulear and Characklis, 1982; La Motta, 1975). La Motta showed that growth of biofilm in an annular reactor with recycle followed a curve that was approximately sigmoidal over a period of 70 hours, and the specific growth rate of the cells was proportional to the initial substrate concentration. Above a certain glucose concentration, further increases in the amount of glucose did not affect the

Rate of glucose uptake at different glucose concentrations

TABLE 3.1:

Glucose concentration g/l	Uptake rate ¹ g/cell	Uptake rate ² g/cell
2.0	3.7×10^{-13}	4.0×10^{-13}
0.5	4.5×10^{-13}	4.5×10^{-13}
0.2	4.0×10^{-14}	4.0×10^{-14}

¹ Approximate uptake rate in the initial linear region

² Approximate uptake rate in the logarithmic region

specific growth rate. The specific growth rate was also directly proportional to the rate of uptake of substrate.

Bryers and Characklis (1981) showed that biofilm accumulation over a period of around 70 hours followed an exponential curve, similar to that seen in Figs 3.4 to 3.6 after the first hour.

3. Number of cells remaining per tube at the end of an experiment

Development of a technique to measure the true numbers of cells attached per tube at the at the end of an experiment was important because the number of labelled cells was used to calculate uptake per cell: thus if division of attached cells occurred during the run period, the calculated uptake per cell would not be accurate. Without a true measure of cell numbers at the end of each experiment, it was necessary to restrict run periods to periods of less than 2 hours to ensure that growth did not occur.

The following methods were investigated:

(i) Total organic carbon (TOC) or cell protein measurements. These methods were not sensitive enough given the low numbers of cells attached to the tubes (Maximum numbers adhered in most experiments was approximately 10^8 cells per tube, and the minimum numbers, in tubes exposed to high shear stresses, was several orders less).

(ii) Lipopolysaccharide (LPS) measurements (Maeda and Taga, 1979). It was felt this method may be too variable in the case of attached cells as there is a wide variation in the amount of LPS produced by bacterial species and strains, and LPS production can be dependent upon the environment and microbial growth rate. Maeda and Taga (1979) showed that in

some cases particulate LPS was related to biomass and in other cases it was related to the numbers of large bacterial cells (length > 1 μ m) when evaluating bacterial standing crop in seawater.

(iii) Adenosine triphosphate measurements (Tobin et al, 1978). This technique has been found to vary in sensitivity (Paul and Loeb, 1983), and for some organisms, ATP per cell has been found to change as growth conditions change, becoming highest during log-phase growth (Jeffrey and Paul, 1986a).

(iv) DNA staining method (Hobbie et al, 1977; Cesarone et al, 1979; Labarca and Paigen, 1979; Paul, 1982; Paul and Loeb, 1983).

(v) Labelling with 3 H Thymidine (Brock, 1967; Hollibaugh et al, 1980; Fuhrman and Azam, 1982; Jeffrey and Paul, 1985).

(vi) Elution of cells from the surface using a surfactant.

The final three methods were thought to be suitable and were tested.

3.1 DNA Staining Technique using Hoechst 33258

This technique was based closely on the method developed by Paul and Loeb (1983). The method is based on the extraction and fluorometric determination of DNA, and is rapid, and very specific and sensitive. In addition the Hoechst 33258-DNA complex which is formed is hardly affected by common laboratory reagents or low concentrations of detergents (Cesarone et al, 1979).

In the case of freely-suspended bacteria, the cells were grown up in glucose medium for 24 hours at 15°C to early

stationary phase. The cells were washed once in phosphate buffer (0.04 M KH_2PO_4 and NaOH buffer, pH 7.4) and resuspended in 10 ml phosphate buffer. Serial dilutions were made up to 1:200, and 0.2 ml samples were taken in triplicate at each dilution and placed in 17 x 100 mm test tubes. The samples were kept on ice. A 3 μl portion of 5% Triton X-100 solution (Sigma Chemical Company Ltd., Poole) was added, and then 1.8 ml of SSC buffer, which contained 0.154 M NaCl, and 0.015 M trisodium citrate in distilled water at pH 7.0. The solution was sonicated for 30 s (MSE 150 Watt Ultrasonic Disintegrator Mk2, MSE Scientific Instruments, Crawley, England) using a 3/8" probe. A 1 ml aliquot of Hoechst 33258 (Aldrich Chemical Co., Gillingham, Dorset) solution at 1.5×10^{-6} M (high range samples) or 1.5×10^{-7} M (low range samples) was added to 2 ml of the extract (brought to room temperature), and the fluorescence measured after 10 min during which time the tubes were kept in low light using a Perkin Elmer LS-5 luminescence spectrophotometer (excitation, 350 nm; emission, 450 nm; slit width, 10 nm for both monochromats).

It was necessary to prepare a fresh stock solution of Hoechst 33258 weekly. A 1.5×10^{-4} M solution was prepared in distilled water, and kept in a dark glass bottle wrapped in aluminium foil at 4°C. This was checked before use: the absorbance should be $4.2 \times 10^{-4} \text{ M}^{-1}\text{cm}^{-1}$ at pH 7.0 (Paul and Myers, 1982). Working solutions of the dye were prepared by diluting the stock solution with SSC buffer to a concentration of 1.5×10^{-6} M for samples with a DNA content of 0.5 to 10 μg , or to 1.5×10^{-7} M for samples with a DNA content of 50 to 750 ng.

The numbers of intact free living cells before and

after sonication were checked using a counting chamber (Weber Scientific Int. Ltd., Lancing, Sussex) at a magnification of 40x (Kyowa optical light microscope).

A stock solution of DNA was prepared by dissolving 5 mg DNA (Calf Thymus, type 1; Sigma Chemical Company Ltd., Poole) in 5 ml SSC, sonicating for 10 s, and stirring magnetically for 30 min. Working stock solutions were made by dilution to 0.1 mg ml⁻¹ for high range standards, of 0.01 mg ml⁻¹ for low range standards. The absorbance of a 1% (w/v) solution should be 174 (Paul and Myers, 1982) at 260nm.

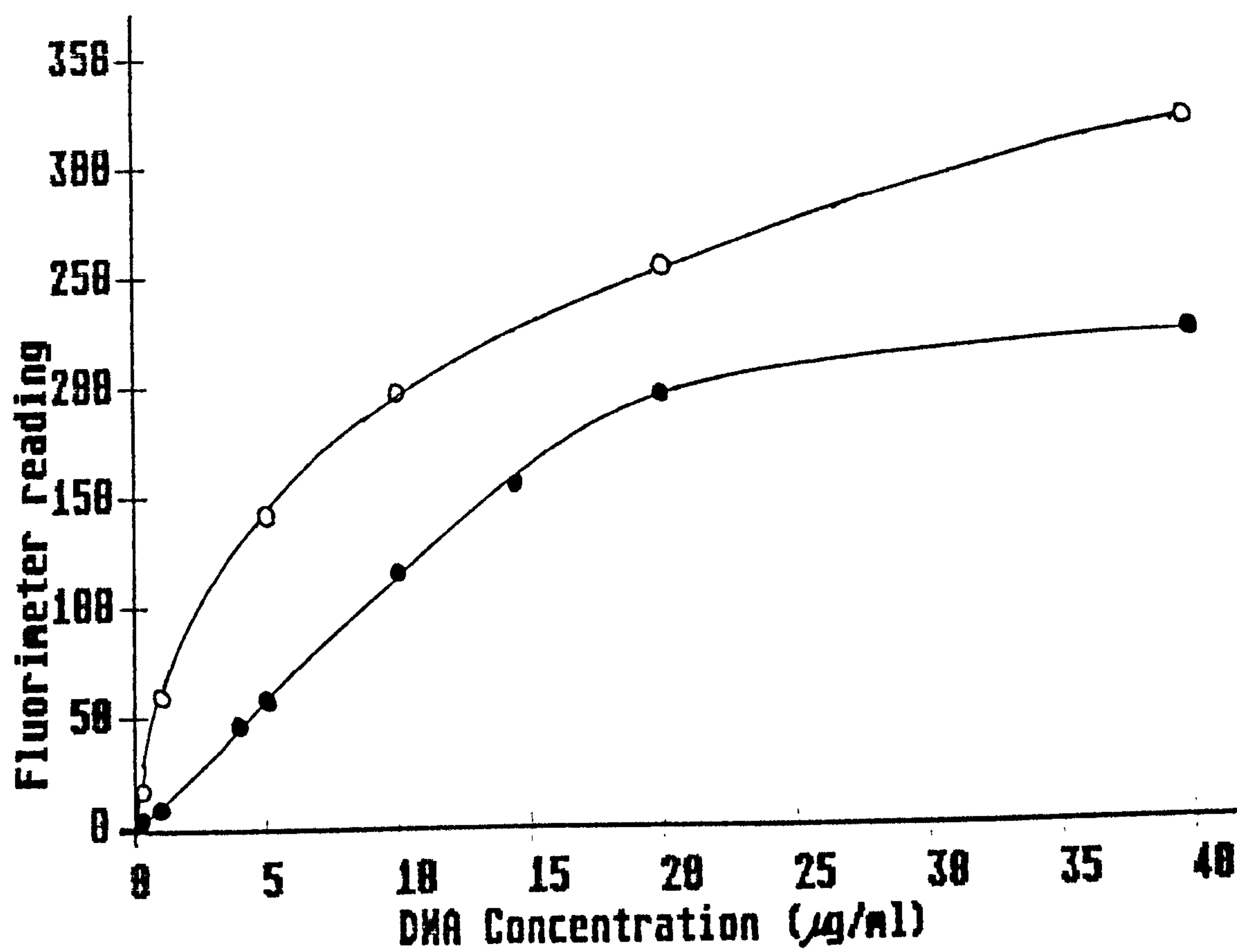
In the case of attached cells, the procedure was modified slightly. The cells were grown up, labelled with ³H-Leucine (specific activity 45.0 Ci/mmol), and attached to the insides of the stainless steel tubes following the methods described on page 74. The leucine label was used to determine the numbers of cells attached to the surface of control tubes which were not sonicated. The tubes were then rinsed 3 times in distilled water and placed in test tubes measuring 6 mm x 75 mm. A 3 µl aliquot of Triton X-100 was added to the tube, followed by 3 ml of ice cold SSC buffer. The tube was kept on ice, and sonicated for 45 s. The sonicator power setting was at approximately 100 W. Two ml of the extract liquid was added to 1 ml of 1.5 x 10⁻⁴ M Hoechst 33258. DNA was determined fluorometrically with calf thymus standards.

In the first two experiments using both suspended and attached bacteria, all the readings from the fluorimeter were between 30 and 50 and there was no correlation between fluorimeter readings and cell numbers. However the fluorimeter readings were found to be correlated with concentration of DNA in calf thymus standards (see Fig 3.7).

3.7 Calibration curve 1 for calf thymus DNA
(Sigma).

Key:

- $1.5 \times 10^{-6} \text{M}$ Hoechst 33258
- $1.5 \times 10^{-7} \text{M}$ Hoechst 33258



Various measures were taken to check whether there were any problems in any part of the procedure.

3.1.1 Improvement of the sonication procedure

It was possible the cells were not being broken up properly during sonication. This was checked and it was found that the majority of cells still appeared to be intact when viewed at a magnification of x 1000 using a light microscope (Kyowa Optical). A series of experiments were carried out to establish the best sonication procedure. The variables which could affect the cell disintegration were the power setting of the sonicator, the sonication time, the volume of sample which was sonicated, and the geometry of the container holding the sample. In addition, there was a possibility that a sufficient temperature rise during sonication could denature the DNA.

The effect of changing the sonicator power setting and varying sonication time was investigated in order to check whether the efficiency of sonication could be improved. At power settings of more than half the maximum possible (maximum power setting was 150 W) foaming occurred, reducing the efficiency of cell disintegration. Power settings were therefore kept to below 75 W. Complete break-up of the cells was not achieved for sonication times up to 20 mins, and long sonication times were not desirable. The effect of sonicating the cells in three 20 s bursts, with the power set at 1/3 of the maximum (approx. 50 W), was tested, and this was found to be the best compromise, giving improved levels of efficiency.

The rise in temperature during sonication was checked using the original power setting and sonication time, and it

was found that the temperature of the sample rose by 5°C. All samples were therefore kept on ice throughout the sonication as a precaution, although it was thought that a temperature rise of 5°C would be unlikely to denature the DNA.

The surfaces were originally sonicated by placing them in test tubes of the same diameter as the stainless steel tubes (6 mm), and positioning the probe tip inside the tube. However it was difficult to prevent the edge of the tube from touching the probe, dissipating its energy. It was found that sonication was more efficient if the tube was cut open using tin-snips and made as flat as possible with fine tweezers. Coupons of 1 cm² were cut out, and these were placed directly under the probe of the sonicator. Almost 100% of the attached cells were removed from the surface during sonication.

The effect of the container geometry on the efficiency of sonication was tested, and it was found that sonication was most effective using bijoux for samples of free-living cells, and 25 ml glass beakers for the surfaces.

The effect of the volume of sample on the efficiency of the sonication was tested by adding varying amounts of SSC buffer to samples. For maximum sensitivity it was necessary to use the smallest volume possible. However, in the case of the free-living samples, it was found that foaming was likely to occur for sample volumes of less than 3 ml, and for attached samples foaming was likely if the volume of liquid was less than 4 ml, hence the volume of the sample was set at these levels.

After carrying out these tests and modifications, the efficiency of sonication was improved such that more than 90% of the cells disintegrated. This was considered to be

satisfactory.

After each improvement in the sonication efficiency, the DNA assay was carried out to check whether there was an improvement in the correlation between fluorescence readings and cell numbers, however there was little change.

3.1.2 Problems with different batches of Hoechst 33258

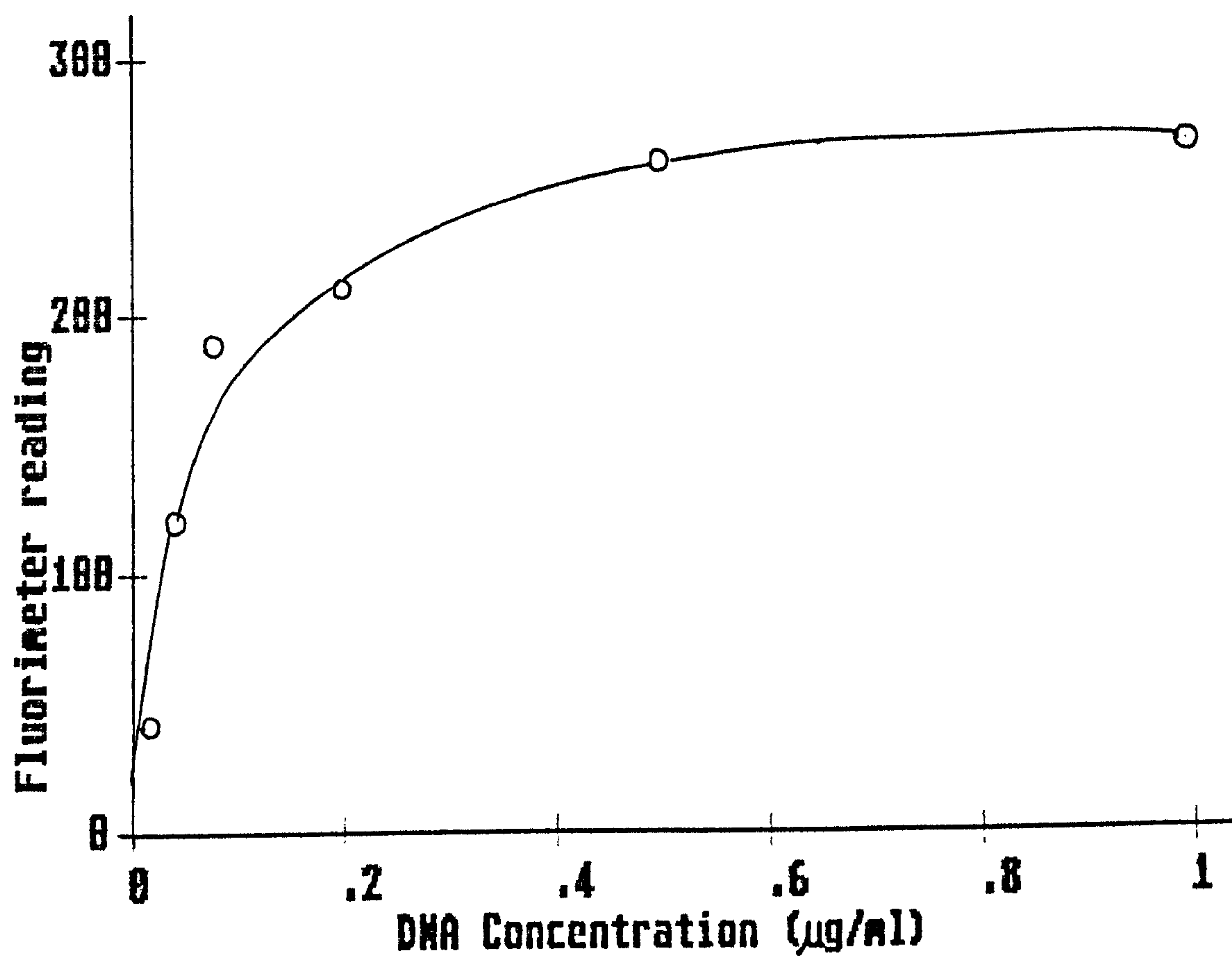
Calf thymus DNA (Sigma Chemical Company Ltd., Poole) solutions were used to calibrate the fluorimeter. Initially a calibration curve was obtained using the Hoescht 33258 from Aldrich (see Fig 3.7). Shortly after the start of work on this technique, however, a new stock of Hoechst 33258 was ordered from a different supplier (Sigma Chemical Company Ltd., Poole). A fresh stock solution was made up using this batch of dye: when the absorbance was checked it was well below $4.2 \times 10^{-4} \text{ M}^{-1}\text{cm}^{-1}$ at pH 7.0. A DNA calibration using the calf-thymus DNA and this stock of dye gave very different results to those obtained with the original stock of dye from Aldrich, and when this was repeated, the results were different from both of the previous calibrations. It was possible that the dye had deteriorated in transit, so a fresh supply was obtained from Sigma. The absorbance of a stock solution prepared using this batch of the dye was slightly too low: however it was much higher than the absorbance of first batch of Hoechst dye obtained from Sigma. The calibration curve obtained was different from any of the previous curves (see Fig 3.8).

A range of dilutions of bacteria were prepared for a DNA assay as described above. Upon addition of the Hoechst dye the fluorimeter readings were between 7 and 26, and decreased with increasing cell concentration in general, though there

3.8 Calibration curve II for calf thymus DNA
(Sigma).

Key:

○ $1.5 \times 10^{-4} \text{M}$ Hoechst 33258



was no relationship between cell concentration and the readings obtained.

At this point, a stock of Hoechst 33258 was ordered from the supplier of the original batch (Aldrich). The absorbance of the stock solution was as it should be. The DNA calibration curve shown in Fig 3.7 was reproduced using this fresh batch of Hoechst 33258. However, in experiments to assay the DNA in cells, the results obtained were as low and random as previously.

3.1.3 Variation of the concentration of detergent

It was possible that the reason why the DNA assays for the cell suspensions were not as expected was due to the very low level of detergent employed (3 μ l of 5% Triton X-100 per 2 ml sample). The sonication may have been breaking up the cells, but not releasing DNA at this level of detergent. The concentration of the Triton X-100 was increased to 0.1%, and an alternative highly efficient detergent, cetyl trimethyl ammonium bromide (CTAB; BDH Chemicals Ltd., Atherstone, Warwicks.) was used at 0.01% and 0.1% concentrations (diluted in SSC buffer).

The initial results for two experiments were encouraging. High fluorescence readings were obtained in the experiments at which the higher concentrations of detergent were used, but it was noticed that the readings tended to drift down over time, in some cases changing quite rapidly. The results still did not show a good correlation between cell number and the fluorimeter reading.

3.1.4 Temperature of samples

It was possible that the drifting of the fluorimeter

readings, which had been occasionally noticed prior to the experiments in which the detergent levels were altered, was due to the fact that the samples were sometimes below room temperature when placed in the fluorimeter. To combat this, the cell sample was brought fully up to room temperature (which was up to 32°C on some occasions), before addition of the dye. This usually took around 2 hours, during which time the samples were kept in the dark. The concentration of the Hoechst 33258 dye was increased tenfold, so that only 0.1 ml of cold solution needed to be added, rather than 1.0 ml, and 0.9 ml of SSC buffer at room temperature was added to keep the sample volume constant.

The readings were still found to be drifting down (see Fig 3.9), and neither the first reading at time zero, or the final reading, taken when the reading had stopped drifting down, bore any resemblance to the number of cells in the sample.

It was then realised that the controls, containing SSC buffer and Hoechst dye, did not contain detergent, and it was found that when controls were prepared with the same amount of detergent solution as the cuvettes containing DNA, very high fluorimeter readings were obtained, in spite of the fact that the sample contained no DNA. It was possible that the detergent itself was in some way affecting the readings.

3.9 Change in fluorescence readings with time.

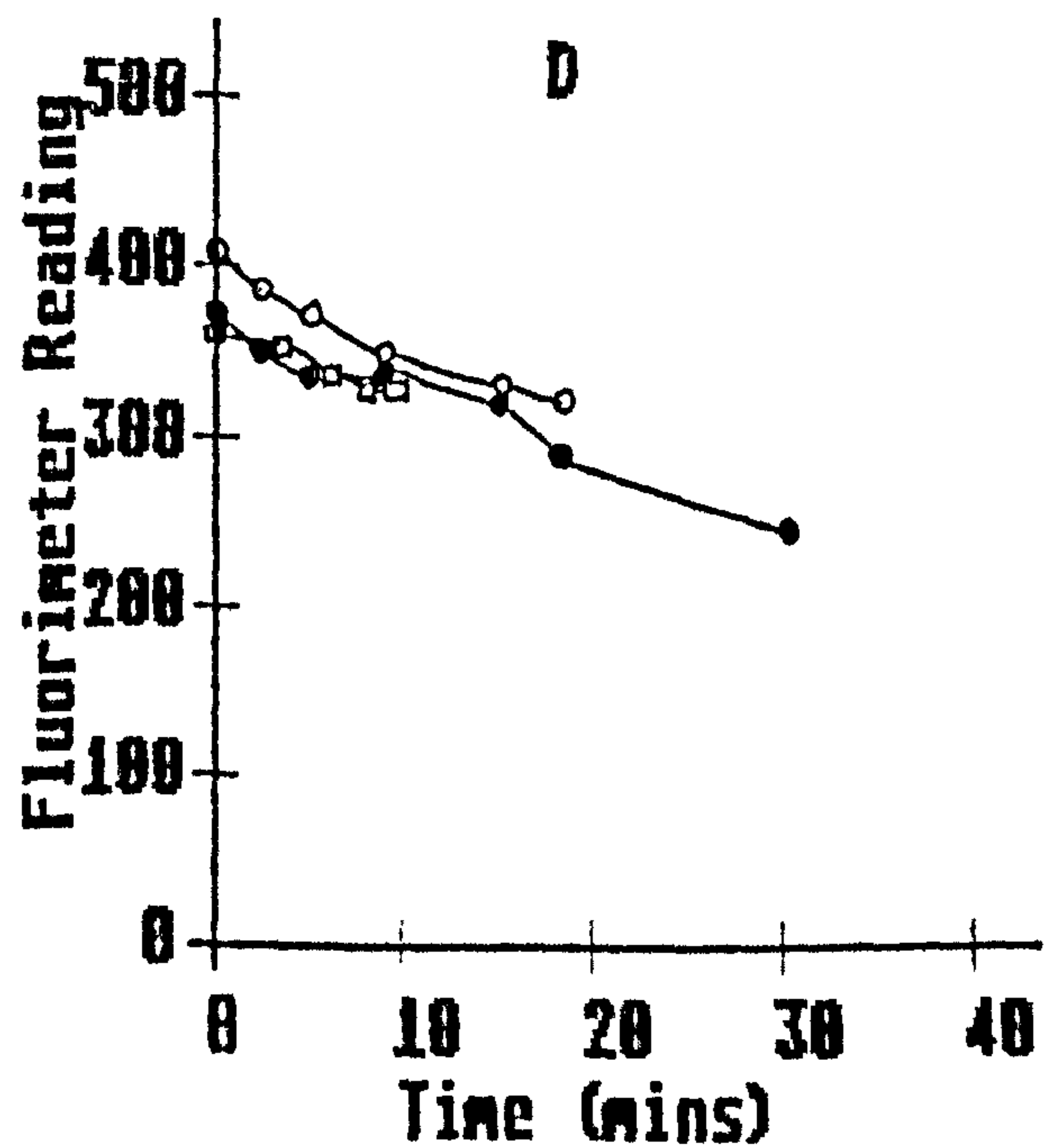
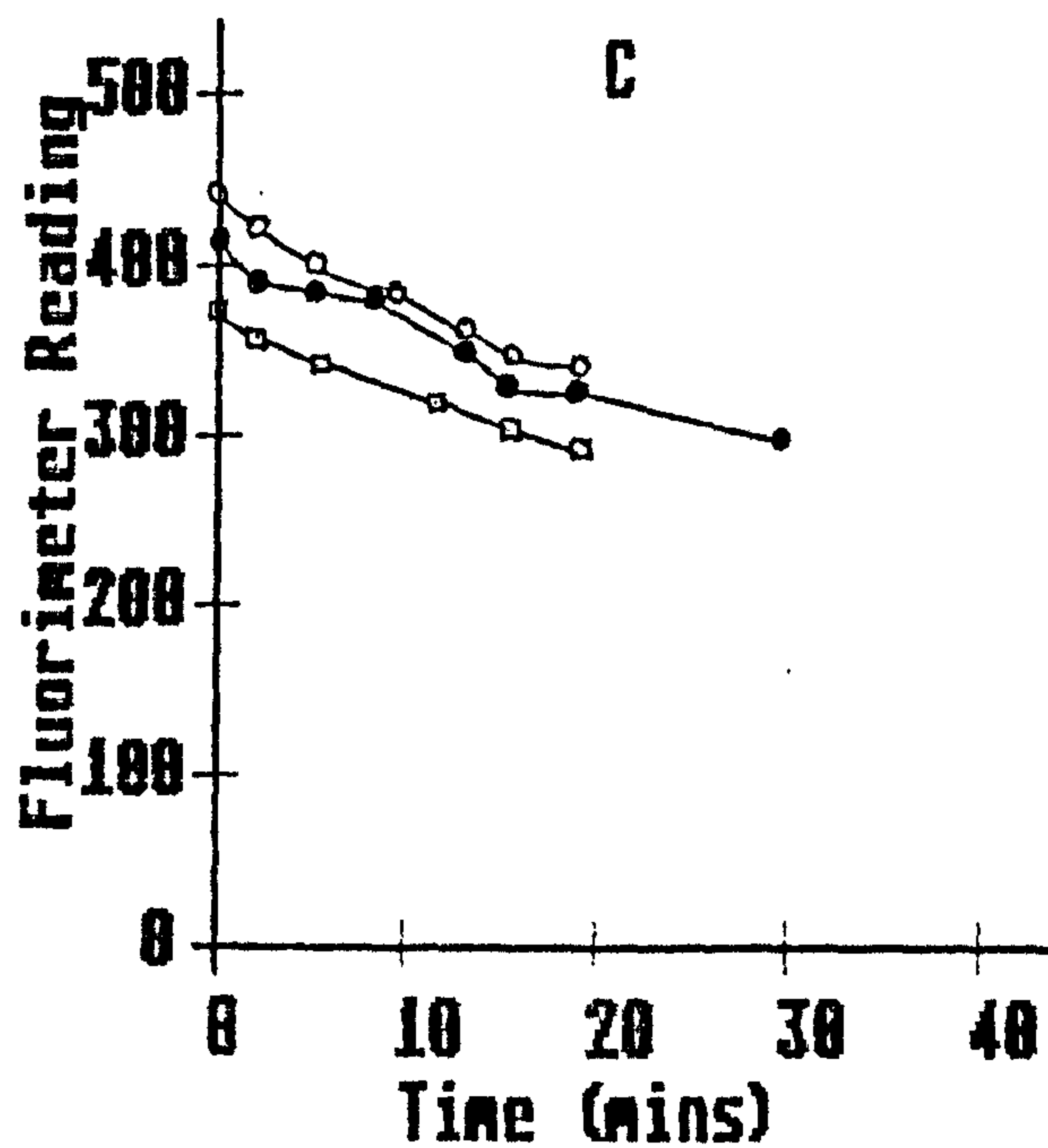
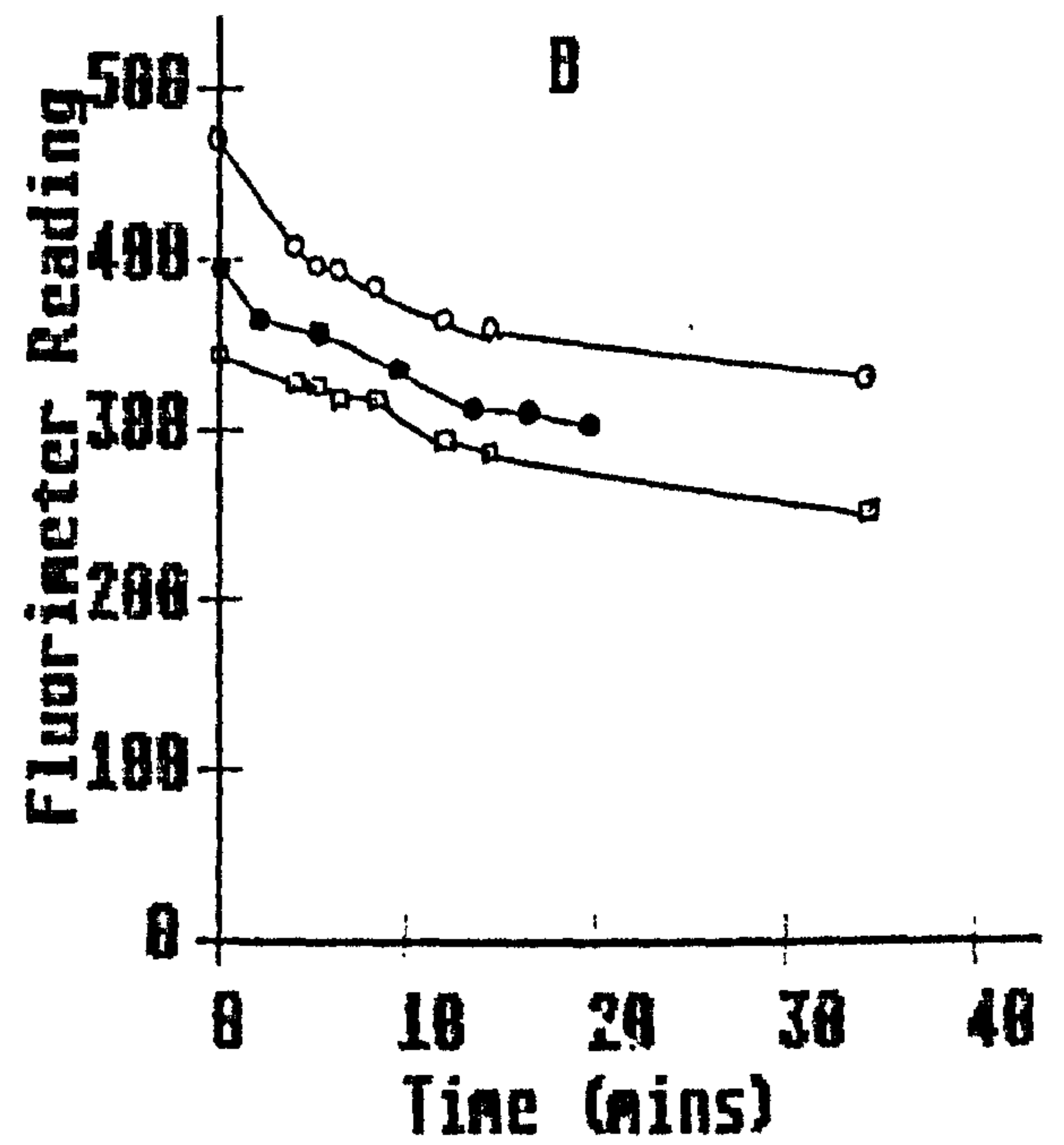
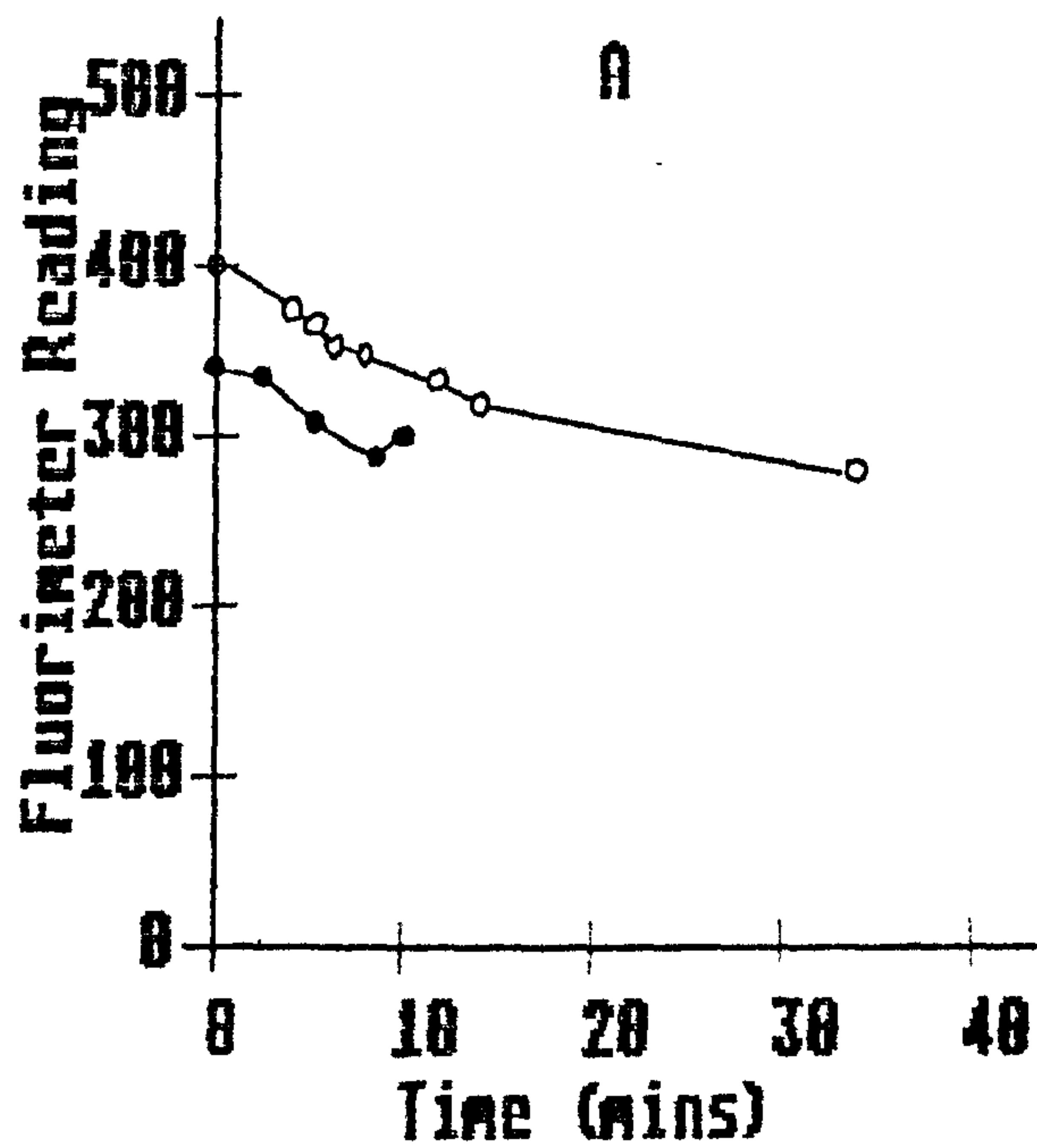
Cell concentration:

A: 2×10^4 cells/ml.

B: 2×10^5 cells/ml.

C: 2×10^6 cells/ml.

D: 2×10^7 cells/ml.



3.1.5 Experiment to check whether the detergent was affecting fluorescence readings

A series of cuvettes were set up, containing calf thymus DNA (2 ml at 0.01 mg/ml, or 2 ml at 0.1 mg/ml) and Hoechst 33254 (0.1 ml at 1.5×10^{-6} M). Aliquots of Triton X-100 (2.5% solution), in the range 6-120 μ l were added, and the total volume of each sample made up to 3 ml using SSC buffer. It was found that the fluorescence reading obtained was directly proportional to the concentration of Triton X-100 for both of the DNA concentrations tested (Table 3.2). The concentration of the Hoechst stain was also varied during these experiments, and found to affect the reading obtained (Table 3.3).

At this point the technique was abandoned. Experiments had been repeated many times and could not be reproduced and in spite of numerous tests and modifications it was still not possible to calibrate numbers of bacteria with fluorescence readings.

Effect of varying amount of detergent (Triton X-100)

TABLE 3.2: Results of 23/7/86

DNA Concentration	TRITON X-100 mls	FLUORIMETER READING
0.01	0.0	15.8 ¹
0.01	0.006	17.7 ¹
0.01	0.06	22.7 ¹
0.01	0.12	176.0 ¹
0.1	0.0	13.3
0.1	0.006	22.2
0.1	0.012	30.7
0.1	0.06	261
0.1	0.12	448

¹ Average of two duplicates. Standard deviations were less than 6% of the mean.

Effect of changing detergent, stain and DNA concentrations on fluorimeter reading

TABLE 3.3: Results of 23/7/86 and 29/7/86.

Concn mg/ml	DNA Volume ml	HOECHST STAIN g/ml	TRITON X-100 ml	FLUORIMETER READING
0.01	2.0	1×10^{-3}	0.12	591
0.01	2.0	1×10^{-3}	0.006	23
0.01	0.0	1×10^{-3}	0.12	561
0.01	0.0	1×10^{-3}	0.006	26
0.1	2.0	1×10^{-4}	0.12	448
0.1	2.0	1×10^{-4}	0.006	22
0.01	2.0	1×10^{-4}	0.12	457
0.01	2.0	1×10^{-4}	0.006	200
0.01	0.0	1×10^{-4}	0.12	304
0.01	0.0	1×10^{-4}	0.006	25
0.01	2.0	1×10^{-5}	0.12	102
0.01	2.0	1×10^{-5}	0.006	115
0.01	0.0	1×10^{-5}	0.12	28
0.01	0.0	1×10^{-5}	0.006	3
0.01	2.0	1×10^{-6}	0.12	11
0.01	2.0	1×10^{-6}	0.006	11
0.01	0.0	1×10^{-6}	0.12	4
0.01	0.0	1×10^{-6}	0.006	1
0.0	0.12	0.0	0.12	1
0.0	0.006	0.0	0.006	0

3.2 Thymidine labelling

It was likely that the addition of tritiated thymidine to the flow rig during an experiment would allow the bacterial production during an experimental run to be calculated. The method is specific for nonphotosynthetic bacteria, and it has been shown that virtually all of the 'active' bacteria in sea water samples take up thymidine (Fuhrman and Azam, 1982). Thymidine is a specific precursor for DNA, which is the only macromolecule that does not turn over and is synthesised only by proliferating cells (Tobin and Anthony, 1978). The method depends on the fact that only dividing cells synthesise DNA, and only during DNA synthesis is tritiated thymidine incorporated into acid-insoluble material (Brock, 1967). The DNA is extracted with trichloroacetic acid.

Depending on the length of time it took for cells to take up thymidine (in 1967 Brock reported that 1% of cells became radioactive per 0.002 generations), there would be a lag in the thymidine uptake versus cell numbers correlation. An experiment was carried out to check whether thymidine was taken up by Pseudomonas fluorescens, and what the uptake time was.

A side-arm flask containing 100 ml glucose medium (preparation described in Chapter 2, page 33) was inoculated with 1 ml Pseudomonas fluorescens culture. After 20 hours growth at 15°C on a rotary shaker at 150 rpm, the culture had reached log phase, (optical density 0.27 measured at 540 nm using a Corning Colorimeter 252), and 20 µCi tritiated methyl thymidine were added (specific activity 86 Ci/mmol). The concentration of tritiated thymidine was thus 2.33×10^{-7} M. At intervals, the absorbance of the culture was checked, and

duplicate 10 ml samples of the culture were removed and filtered using cellulose nitrate filters (2 μ m pore size; Sartorius). The filters were washed twice with 5 ml of distilled water and placed in glass scintillation vials containing 10 ml Optiscint 'safe' scintillant (LKB Instruments Ltd.). These were counted in a scintillation counter (System LS-7000, Beckman Instruments Inc., Fullerton, Calif.) using Programme 5 (for tritium-labelled samples).

It was thought that the low counts obtained for the first four samples could be due to the fact that the concentration of labelled thymidine was too low for the cells to 'see' it. One ml of unlabelled thymidine at a concentration of 15.4 mg/l was added to the culture in order to achieve a final concentration of 1.06 M.

It was also possible that the counts for the first four samples (taken at 30 min intervals) were low due to a problem with the scintillant. A 1 ml aliquot of tissue solubiliser (Optisolv, LKB Instruments Ltd.) was added to check whether this affected the counts as the scintillant used, although suitable for use with filter discs, was not suitable for measuring aqueous samples. When the vials were recounted after the addition of the tissue solubiliser the counts were increased; however it was found that the scintillant was not compatible with cellulose nitrate filters.

For the subsequent samples, taken at intervals up to 6 hours after the addition of labelled thymidine, 3 samples of 1 ml were taken. Two of the filters were put straight into 10 ml scintillant, as before, and the remaining filter was put into 10 ml scintillant with 1 ml ethanol added.

From the results (some of which are summarised in Table 3.4), it appears that the label was not being taken up by the cells, even after addition of unlabelled thymidine. As the tissue solubiliser was incompatible with the filters these results can be disregarded. The addition of ethanol increased the counts but the counts did not show a direct relationship with cell numbers (it was possible the ethanol was causing chemiluminescence, but this seems very unlikely).

Another more comprehensive experiment was carried out, in which two different amounts of labelled thymidine and four concentrations of unlabelled thymidine were added to glucose medium, which was then inoculated with bacterial culture. The scintillant used (Optiphase 'safe'; LKB Instruments Ltd) was suitable for aqueous samples and samples on filters.

An aliquot of 20 ml glucose medium (made up as described above) was placed into each of six 50 ml flasks. Thymidine and labelled thymidine were then added to the flasks as detailed in Table 3.6. One ml of Pseudomonas fluorescens culture was inoculated into each flask.

At intervals, 3.4 ml samples were withdrawn from each flask. This was distributed as follows: (i) two 50 μ l aliquots were placed in glass vials containing 10 ml scintillant; (ii) duplicate 1 ml aliquots were filtered and the filters were washed twice with 5 ml distilled water before being added to glass vials containing 10 ml scintillant, and

Uptake of thymidine by a growing culture

TABLE 3.4: Results of 6/8.

Time after addition of ³ H Thymidine ¹ (mins)	Optical density	Volume Filtered (mls)	Counts per minute ²	Contents of vial
0	0.27	10	289	S
0	0.27	10	39143	S + TS
30	0.30	10	339	S
30	0.30	10	84263	S + TS
60	0.36	10	297	S
60	0.36	10	59800	S + TS
230	0.53	1.0	292	S
230	0.53	1.0	4558	S + E
230	0.53	50 µl filtrate	8265	S + E
270 ³	0.59	1.0	746	S
270	0.59	1.0	4743	S + E
360	0.71	1.0	293	S
360	0.71	1.0	4574	S + E

¹ Some of the results for intermediate times are omitted.

² The mean value for the two duplicates is shown. The standard deviation for the duplicate vials were less than 10% of the mean.

³ Unlabelled thymidine was added at this time.

KEY:

S - scintillant
E - ethanol
TS - tissue solubiliser

(iii) the absorbance of the remainder of the sample was determined spectrophotometrically at 540 nm (SP-1700 spectrophotometer, PYE Unicam Ltd., Cambridge), using 1 ml glass cuvettes. The vials were counted on the tritium channel (Programme 5) in a liquid scintillation counter (System LS-7000, Beckman Instruments Inc, Fullerton, Calif.).

Table 3.6 shows that tritiated thymidine was incorporated into cells; however, the thymidine uptake showed no correlation with either the cell growth, or the concentration of thymidine provided (Tables 3.5, 3.6 and 3.7).

Table 3.5 shows the growth of the culture, as measured by increase in absorbance, over the time period of the experiment.

Table 3.6 shows the amount of tritiated thymidine in the whole culture and shows that none is lost in 43.5 hours. The counting efficiency is approximately 25%.

Table 3.7 shows the thymidine taken up by the filtered cells. Some thymidine would appear to have been taken up (less than 1.5% of the added tritiated thymidine), but the amount was not related to the growth of the cells.

Increase in absorbance of culture

TABLE 3.5: Results of 12/8/86.

Time hours)	Average absorbance ¹	Standard deviation
0.5	0.01	0.0
19.0	0.89	0.23
24.0	1.40	0.06
43.5	1.64	0.02

¹ Averaged for the 6 flasks.

Counts obtained for 50 µl culture

TABLE 3.6: Results of 12/8/86.

Unlabelled thymidine concn in flask	Total ³ H labelled thymidine in flask	CPM FOR 50 µl OF CULTURE			
		0.5 h	19 h	24 h	43.5 h
10 ⁻⁴ M	10 µCi	13150	13088	12157	12344
10 ⁻⁵ M	10 µCi	12787			13041
10 ⁻⁶ M	10 µCi	12688			12722
10 ⁻⁷ M	10 µCi	12752			12721
10 ⁻⁴ M	2 µCi	2973	2480	2796	2834
10 ⁻⁶ M	2 µCi	2819			

Scintillation counts for filtered cells

TABLE 3.7: Results of 12/8/86.

Unlabelled thymidine concentration in flask	Total ^3H labelled thymidine per flask	CPM FOR FILTERED CELLS			
		0.5 h	19 h	24 h	43.5 h
10^{-4} M	10 μCi	3341	2365	1196	2047
10^{-5} M	10 μCi	2375	2403	1125	2036
10^{-6} M	10 μCi	2528	3666	1471	2876
10^{-7} M	10 μCi	2294	1295	1200	2230
10^{-4} M	2 μCi	491	350	622	471
10^{-6} M	2 μCi	398	515	-	-

3.3 The use of various surfactants to remove cells from surfaces without loss of viability

The intention of this study was to investigate a technique for removing cells from surfaces in a viable condition, in order to estimate the numbers of cells at the end of an experimental run by standard serial dilutions and plate counts.

Tween 20 has been shown to remove viable cells from stainless steel surfaces enabling evaluation of the numbers of cells attached to the surface (personal communication, Dr S Y R Pugh). An outline of the method used to check this is given below, followed by details of the optimisation of various features of the method.

A culture of Pseudomonas fluorescens was grown up to early stationary phase in glucose medium (prepared as described in Chapter 2, page 33) for 24 hours in a shaking incubator at 15°C. The cells were labelled with ³H-leucine (for description of labelling method see Chapter 2, pages 68-74). This was done so that the number of cells attached to the tubes could be established.

The cells were attached to the insides of the 5 cm stainless steel tubes using the attachment system. Up to this point the procedure was identical to that followed in experiments involving the test-rig.

The tubes were rinsed 3 times in distilled water, drained lightly by holding the tube vertically onto a piece of absorbent tissue. Some of the tubes were kept aside so that the numbers of cells which were attached to the tubes could be measured (see below). The remainder were placed in test tubes containing 3 ml surfactant solution. This solu-

tion was made by adding 50 ppm Tween 20 to 5.44 g/l KH_2PO_4 solution, adjusted to pH 7.4 using 1 M NaOH. These tubes were then bunged tightly and incubated under various conditions (see below).

The stainless steel tubes were then removed from the surfactant solution and rinsed 3 times in distilled water before being dried. They were then kept aside so that the numbers of cells attached to the surfaces could be measured (see below). The surfactant solution, containing the eluted cells, was used to determine the number of cells eluted from the surfaces. The total number of cells present, and the number of viable cells which were eluted by the surfactant solution, were assessed by various methods.

The total numbers of cells present in each test tube was counted using a counting chamber (Weber Scientific Int. Ltd., Lancing, Sussex). However it was found that the number of cells counted was too low to allow any useful comparison of the tubes to be made. The total number of cells present in each tube was also counted using a scintillation counter (System LS-7000; Beckman Instruments Inc., Fullerton, Calif.). This was done using 2 methods. In the first experiments, a small aliquot of the eluted cell solution was added to Optiphase 'MP' scintillant (LKB Instrments Ltd.) and counted: the counts were found to be rather low, so in subsequent experiments, 2 ml aliquots were filtered and the filters were added to 10 ml scintillant.

The numbers of viable cells were found using the spread plate method. An aliquot of the surfactant solution was added to phosphate buffer, and a series of dilutions were prepared from this. A range of three successive dilutions was plated

out. A sterile pipette was used to inoculate glucose-agar with 0.1 ml of the appropriate dilution, and the drop was spread over to surface of the agar using a sterile glass spreader. Three replicate plates at each dilution were prepared. The plates were incubated at 30°C until colonies had grown to a suitable size for counting (about 48 hours). Glucose-agar was prepared by adding 2 g of glucose to each litre of nutrient agar. The glucose was added to the agar as it was cooling.

The stainless steel tubes were treated in various ways. Some were heat fixed at 160°C for 5 minutes, stained with acridine orange (BDH, Atherstone, Warwicks.), cut open, and fixed to microscope slides. The number of cells was assessed by counting using an epifluorescent microscope (Zeiss Standard 18 Microscope, using blue incident light excitation). Other replicate tubes were put into 2.3 ml tissue solubiliser (Optisolv; LKB Instruments Ltd.) after being dried, and following incubation at 50°C overnight (approx. 16 hours), the tissue solubiliser was added to 10 ml scintillant (Optiscint) in glass vials, and counted in the scintillation counter using Programme 5 (for tritium labelled samples). These two methods were used to determine the numbers of cells remaining on the surface after elution with Tween 20 solution. The numbers of cells on the tubes which were not treated with Tween 20 were also measured using these methods.

3.3.1 Preliminary experiments to establish the best method of agitating the tubes during the incubation period

Two methods were compared:

(a) The tubes were whirlmixed for 2 minutes.

(b) The tubes were allowed to roll horizontally on a flat surface for 1 hour in a rotary shaker at 175 rpm and at 15°C.

(A mechanical tube roller which would work satisfactorily for test tubes of this size could not be obtained. However the tubes rolled constantly and evenly on a flat surface in a shaking incubator).

The total number of cells removed from the stainless steel tubes was found using the methods described previously (see Chapter 2). In the case of cells attached to tubes which were measured using a scintillation counter, the percentage elution was calculated by dividing the counts per minute obtained for the tube after incubation with the counts per minute obtained for control tubes. The percentage elution for tubes which were counted microscopically was calculated similarly using the numbers of cells per tube (see Tables 3.8 and 3.9).

It was found that different results were obtained, depending on the method used to calculate the percentage elution. The percentage elution was higher when calculated using the microscopic method. The percentage elution was greater in the case of tubes which were incubated for 1 hour in the surfactant solution.

The percentage of cells eluted by a 50 ppm Tween 20 solution,
measured using an epifluorescent microscope

TABLE 3.8: Results of 11/11/86.

Tube	Cells per tube	Standard deviation	Number of tubes	Percentage Elution
Control	2.14×10^7	2.6×10^6	5	0
Whirli-mixed	1.49×10^7	1.5×10^6	2	28.7
1 hour	1.18×10^7	5.6×10^6	3	54.7

The percentage of cells eluted by a 50 ppm Tween 20 solution,
measured by a scintillation counter

TABLE 3.9: Results of 11/11/86.

Tube	Average disintegrations/minute	Standard deviation	Number of samples	Percentage elution
Control	16930	685	3	0
Whirli-mixed	16258	1880	2	4.0
1 hour incubation	13606	293	2	19.6

The plate counts for three tubes which were incubated for 1 hour with the surfactant solution showed that the average number of cells eluted per stainless steel tube was 9.69×10^6 (standard deviation was 26% of the mean). Using the epifluorescent microscope the average number of cells which were left on the stainless steel tube after the 1 hour incubation was found to be 1.18×10^7 (Table 3.8). If the numbers found by counting plates are added to this (ie addition of cells remaining on tube plus viable cells eluted from tube), the total number originally on the tube can be found. This disregards non-viable eluted cells.

Cells on tube + Viable eluted cells

$$1.18 \times 10^7 + 9.69 \times 10^6 = 2.15 \times 10^7$$

The number of cells counted on the control tubes was 2.14×10^7 , so there was a very close agreement, suggesting that practically all the eluted cells must have been viable. If this approach is taken using results counted in the scintillation counter, it would suggest that some of the cells were non-viable.

Aliquots of the eluted cells were added to scintillant, but the counts obtained were too low to give any useful information. In further experiments a larger aliquot was filtered and counted in the scintillation counter. The eluted cells were also counted using a counting chamber, and the same problem was encountered.

It was concluded that the technique was worth further investigation using a one hour incubation time in surfactant solution; that a large proportion of the eluted cells are possibly viable, and that there was some discrepancy between

the percentage eluted cells estimated by different methods, which required further investigation. Further work was necessary to establish whether the percentage of cells eluted could be increased, and whether the technique was reproducible.

3.3.2 Investigation of a number of different surfactants

The aim of these experiments was to find the most efficient and consistent surfactant in order to remove cells viably from surfaces. The following surfactants were considered: Tween 20 at 50 ppm, Ethylene Glycol Bis-(B-Aminoethyl ether) N,N,N',N-tetra-acetic acid (EGTA) at 0.01 and 0.002 mg/100 ml, and cetyl trimethyl ammonium bromide (CTAB; BDH Chemicals Ltd., Atherstone, Warwicks.) at 250 ppm. The various surfactants were diluted in 5.44 g/l KH_2PO_4 buffer (adjusted to pH 7.4 using 1 M NaOH) to the required concentration. The cells were labelled and attached to stainless steel tubes as previously described. The incubation with surfactant was for 1 hour at 25°C.

The variability in some of the results appears to be closely related to the temperature in the shaking incubator while the tubes were incubated with surfactant. On 26/11/86, the thermostat on the shaking incubator was operating at 39°C. After 25 minutes the tubes were removed to an incubator at 25°C. It can be seen that the percentage elution, as measured by the microscopic method, was greater on this occasion (Table 3.9). On 22/1/87, the tubes were incubated at 15°C. The lower temperature resulted in a lower percentage elution (Tables 3.9 and 3.10). It is therefore extremely important to ensure that the temperature is always at 25°C to ensure reproducible results.

The percentage of cells eluted by various surfactant solutions, measured using an epifluorescent microscope

TABLE 3.10

Date	Tween 20 50ppm	EGTA 0.002 mg/100ml	EGTA 0.01 mg/100ml	EGTA & Tween 20	CTAB 250ppm	H ₂ O
13/11/86	-	58.3	63.1	-	21.4	7.7
26/11/86	64.1	66.9	80.0	89.0	-	-
10/12/86	73.1	57.7	59.4	69.0	-	-
22/1/87	46.7	45.7	54.7	54.3	-	-
AVERAGE	61.3	57.2	64.3	70.8	21.4	7.7
STD DEVN	13.4	8.7	9.5	14.2	-	-

The percentage of cells eluted by various surfactant solutions, measured by a scintillation counter

TABLE 3.11.

Date	Tween 20 50ppm	EGTA 0.002 mg/100ml	EGTA 0.01 mg/100ml	EGTA & Tween 20	CTAB 250ppm	H ₂ O
13/11/86	-	46.2	46.3	-	32.6	5.8
26/11/86	61.9	54.6	60.3	74.4	-	-
10/12/86	61.0	55.6	60.2	74.7	-	-
22/1/87	*	14.4	33.1	14.3	-	-
AVERAGE	61.5	42.7	50.0	54.5	32.6	5.8
STD DEVN	0.6	19.3	13.0	34.8	-	-

* In this case there was no measurable elution: the control tubes had slightly fewer cells attached than the tubes treated with Tween 20. Therefore an overall average elution could not be calculated.

The percentages of cells eluted using a given surfactant in the various experiments fell within a range which appeared to be roughly constant. Thus it was possible that this technique could be adapted for finding numbers of cells remaining attached to the stainless steel tubes at the end of an experimental run, providing that the percentage elution was checked on each occasion.

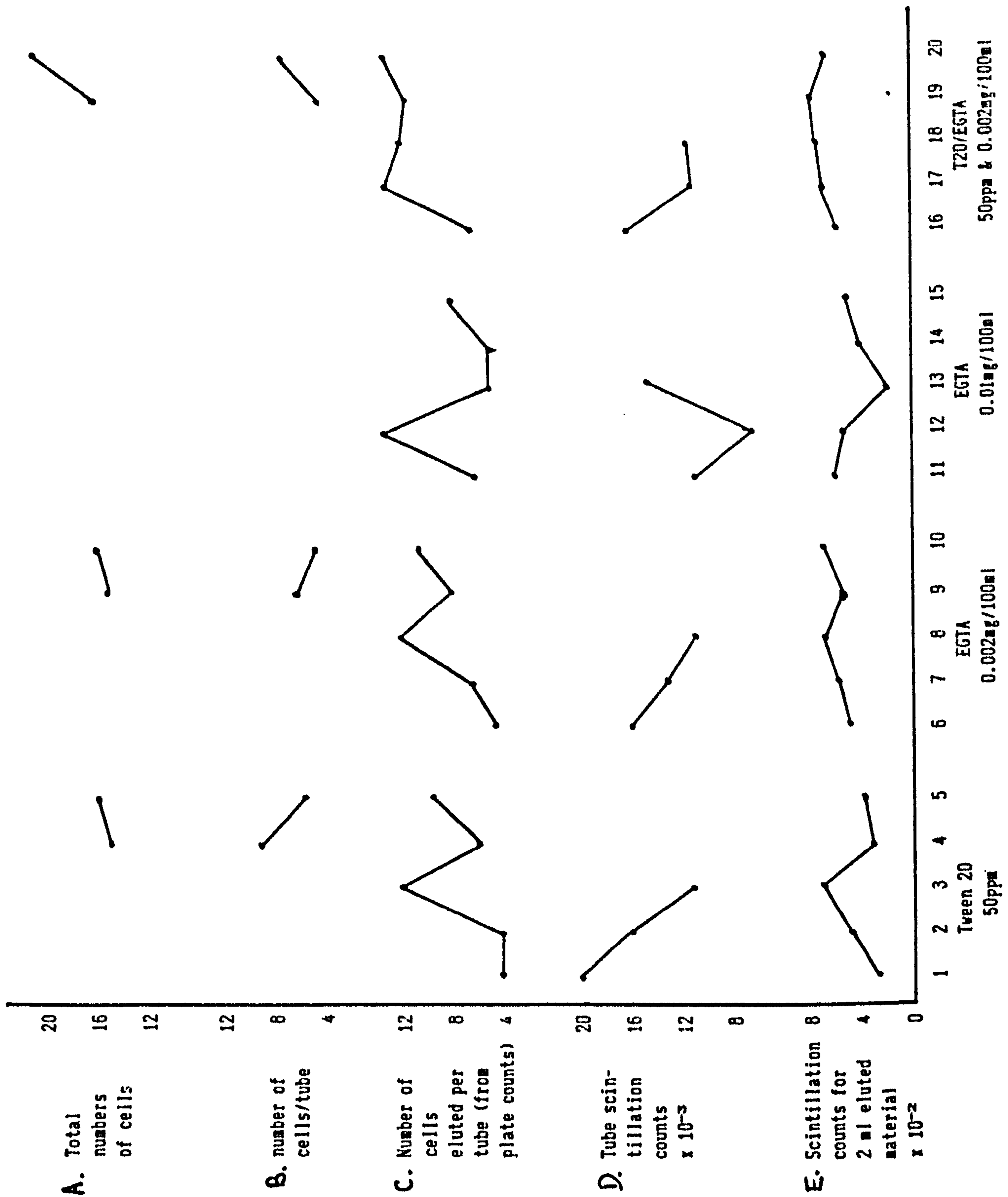
There was a lot of variation in the results for the individual tubes. Fig 3.10 shows the variability in the numbers of cells eluted from, and remaining attached to a series of individual tubes. The top section of the figure shows the actual numbers of cells remaining attached per tube (line B, counted using an epifluorescent microscope), and numbers of cells eluted per tube (line C, from plate counts) for each individual tube. These have been added to show the original total number of cells attached to each tube (line A).

As the percentage of cells which were eluted varied considerably from tube to tube, a considerable number of replicate tubes would be required if this method was to be used to accurately assess the number of cells per tube.

The cells used in these experiments were radiolabelled. The lower section of Fig. 3.10 shows the scintillation counts for the tubes (line D), and for 2 ml aliquots of eluted cell suspension (line E). These show the same trends as the direct measurements of numbers of cells. (Actual numbers of cells have not been shown: however, the scintillation results confirm the variability in the levels of elution.)

3.10 Numbers of cells eluted with time.

- Notes:
1. Individual tubes were labelled 1-20. These are shown on the x-axis.
 2. Lines B and D: it was not possible to make both these measurements for an individual tube.
 3. See page 122 for further notes on this figure.



The technique seemed promising if used with caution. The use of the combination of 0.002 mg/ml EGTA and 50 ppm Tween 20 seemed to give the highest results for elution. However, the level of elution varied from one experiment to another, and temperature also appeared to affect the elution level quite considerably. Thus, it was essential to check percentage elution, following the method above, every time the method was used for finding numbers of attached cells on the tubes at the end of a run in the flow rig, so that any variations between experiments could be assessed.

3.3.3 The use of the surfactant technique to determine numbers of cells on tubes at the end of an experiment using the flow rig

Four experiments were carried out using the flow rig to test the surfactant technique as a method for determining the number of cells remaining on the tubes at the end of an experiment. In each experiment, four of the tubes from each rig were treated as before, (put into tissue solubiliser at the end of the run), whereas five of the tubes were incubated in the surfactant solution for one hour at 25°C, prior to being treated in the same way as the other four tubes.

It was found that there was no significant difference between numbers of attached cells (as counted using an epifluorescent microscope or the scintillation counter) for either set of tubes. This was also backed up by the plate counts; in fact practically no cells were eluted by the surfactant from tubes which had been in the rig. However, in the case of the control tubes which had not been in the rig (treated in the same way as described on p 70-74), the level of elution of cells was in the range previously established.

This implies that cells attached to the tubes which had been in the rig were in some way more firmly attached. The method was therefore useless for determining the number of cells at the end of a run.

Because the time spent in establishing a technique to determine of the number of cells at the end of an experimental run had already been excessive, further search for a suitable method was abandoned, and experimental run periods were kept to short times of less than an hour, during which time it could reasonably be assumed that cell growth (reproduction rather than enlargement) was minimal. This was supported by the fact that few dividing cells were seen when observed using an epifluorescent microscope.

4. Uptake of glucose by free living cells

It was necessary to study the uptake of glucose by freely suspended cells in order to compare the uptake of attached cells with those in suspension. As far as possible the procedure for growing up cells for this experiment was kept the same as for the experiments involving the flow rig. The cells were grown in glucose medium and labelled with ^3H -leucine as described on pages 68-73. After the final resuspension, the cells were resuspended in buffer, and diluted to a concentration of 3×10^6 cells/ml.

Flasks containing 30 ml glucose medium were set up. Six glucose concentrations were tested: 10.0, 2.0, 0.5, 0.2, 0.05 and 0.02 g/l. Three μCi ^{14}C glucose was added to each flask, and at each concentration three replicate flasks were prepared. One ml of the diluted cell suspension was added to each flask, producing a cell concentration of 1×10^7

cells/ml. These flasks were placed in an rotary shaker at 15°C, and 3 x 2 ml samples were taken at intervals from each flask. Two of these were filtered using 1 µm pore size polycarbonate filters, and the filters were placed into vials containing 10 ml Optiphase scintillant. These vials were then counted in the scintillation counter. The remaining 2 ml sample was placed in a 1 ml cuvette and absorbance measurements were made to check whether growth was occurring.

Glucose uptake, in terms of g/cell, was determined as follows:

(a) The efficiency of counting ^{14}C was 78%; thus for a 2 ml sample,

$$\frac{\text{Dpm}}{\text{ml}} = \frac{((\text{cpm}) - (\text{control cpm}))}{(2 \times 0.78)}$$

(b) The ^{14}C glucose concentration was 0.1 µCi/ml, thus

$$\frac{\text{total } ^{14}\text{C glucose uptake (µCi/ml)}}{\text{total } ^{14}\text{C glucose available per ml (µCi/ml)}} = \frac{\text{glucose uptake (g/ml)}}{\text{total glucose available per ml (g/ml)}}$$

(c) Uptake per cell was then found by dividing glucose uptake (g/ml) by the number of cells per ml. The results are shown in Fig 3.11. These results are compared with the results for biofilms in the flow rig in Chapter Five.

Fig 8.4, (given in Appendices, page 201) shows glucose uptake by a growing culture in 2.0 g/l glucose medium. The uptake rate for cells growing in the log phase was approximately 1×10^{-13} g/cell in one hour. This value agrees well with the uptake rate of 1.4×10^{-13} g/cell in one hour measured in this experiment.

3.11 Uptake versus time for freely suspended
 cells.

A: 10.0 g/l glucose

 2.0 g/l glucose

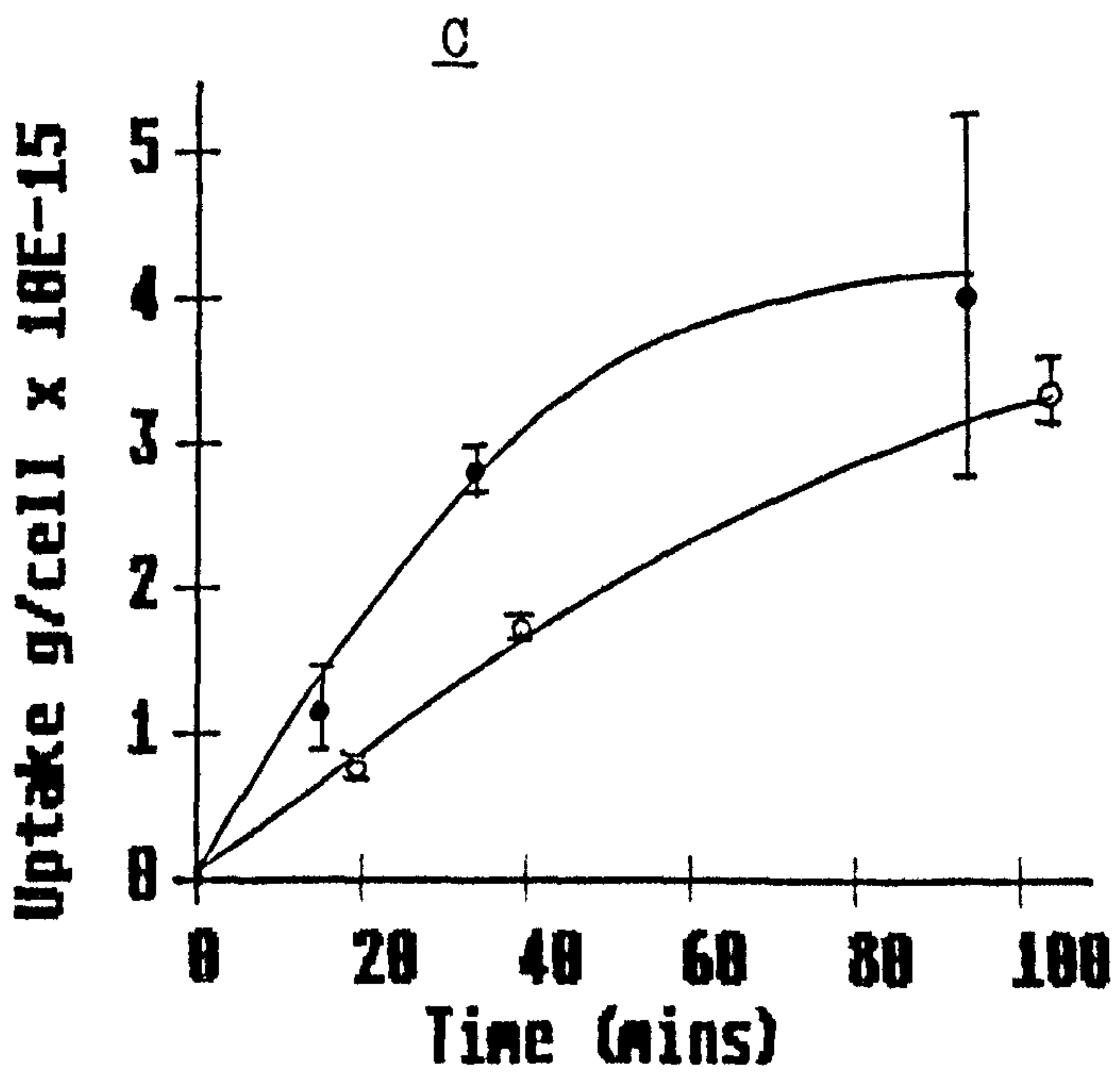
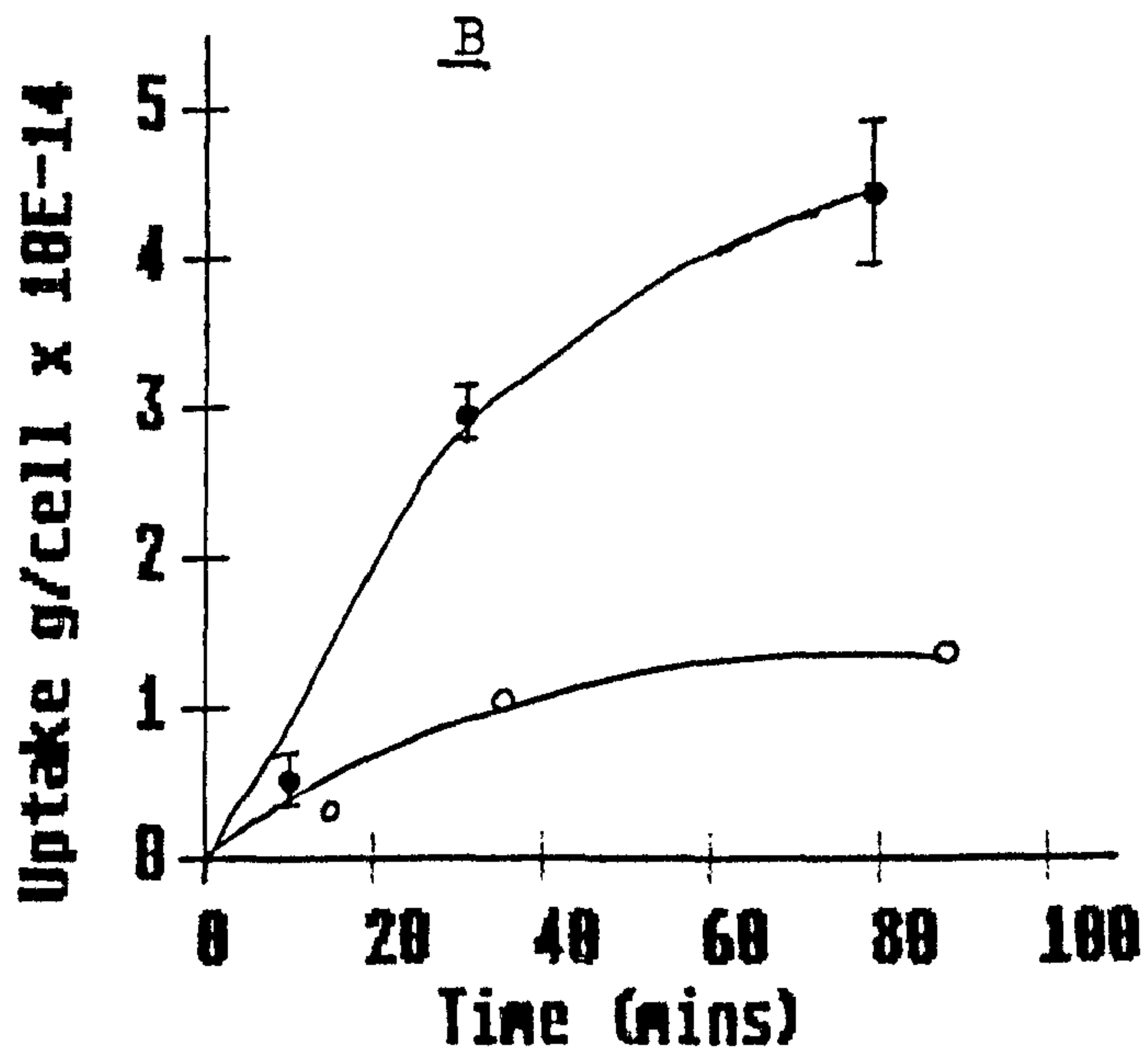
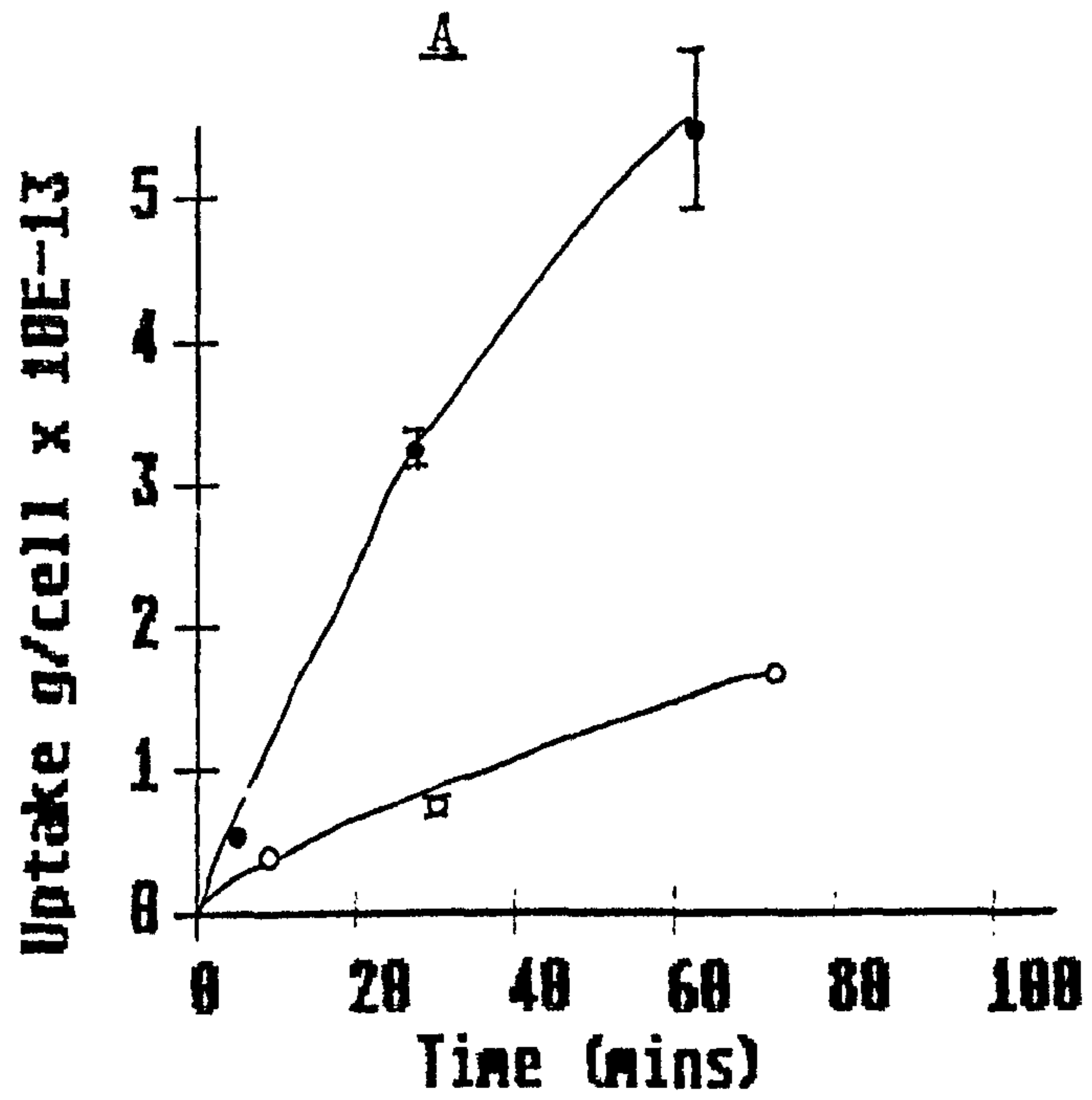
B: 0.5 g/l glucose

 0.2 g/l glucose

C: 0.05 g/l glucose

 0.02 g/l glucose

Note: Where no error bar is shown, the point is derived
from a single result.



4.1 Glucose uptake rates for Pseudomonads

Glucose uptake for Pseudomonas fluorescens was measured by Eisenberg et al (1974): initial uptake rates were shown as a plot against glucose concentration. Uptake rate for the glucose concentration 111 μM (0.02 g/l) was shown to be approximately 13 nmoles glucose $\text{min}^{-1} \text{mg}^{-1}$ cells. In the present study, the average uptake at this concentration, over the first 30 minutes, was 1.3×10^{-15} g/cell (1.2 nmoles glucose $\text{min}^{-1} \text{mg}^{-1}$ cells). This is lower than that measured by Eisenburg et al. There are two principal reasons which may account for this:

(i) Eisenberg et al measured the initial uptake rate over 30 seconds: however, data was presented in the same paper which showed that the initial uptake rate was at least twice the average uptake rate measured over 30 minutes.

(ii) uptake rates were measured at 15°C in the present study, rather than at 30°C, as in Eisenberg's study. Data presented by Lynch and Franklin (1978) show that uptake rates are approximately 3 times lower at 5°C than at 30°C. Thus, the uptake at 15°C is likely to be considerably lower than at 30°C.

Taking these points into consideration, it can be seen that the uptake rates measured in this experiment are likely to be very similar to those measured by Eisenberg et al. Uptake rates measured for Pseudomonas aeruginosa are rather lower than those measured by Eisenberg et al for Psuedomonas fluorescens: for instance, data presented by Eagon and Phibbs (1971) show the glucose uptake rate, at a concentration of 50 μM , to be about 1.0 nmoles $\text{min}^{-1} \text{mg}^{-1}$ cells (compared with an

uptake of about 10 nmoles min⁻¹ mg⁻¹ cells measured by Eisenburg et al). This glucose concentration (50μM) was just below the lowest concentration used in the present study, and so these uptake rates cannot be directly compared.

CHAPTER FOUR
THE EFFECT OF SHEAR STRESS

1. Introduction

The interrelationship between physiological activity, attachment ability, growth on a surface, and flow rate is apparently very complex, and one of the main aims of this study was to obtain more information about some of these links, particularly the effect of flow rate on the 'activity' of the cells.

In previous studies, various parameters have been selected which provide an indication of the metabolic processes, or activity of attached cells. These include changes in the numbers of suspended or attached cells; changes in cell size; respiration rate, measured as carbon dioxide production; oxygen uptake, or intensity of electron transport system activity; substrate uptake or breakdown; product formation and heat production. Some of these are related to one another.

In this study, uptake of glucose was used to evaluate whether the activity of attached cells varied with shear stress. In addition, the uptake per cell was compared to that obtained for suspended cells under the same conditions.

A wide range of flow rates could be obtained using the experimental flow rig. The Navier-Stokes equations, which describe the flow of fluids in any given situation, reduce to a simpler form when inertial effects predominate over viscous effects, or vice versa, in dictating the flow pattern through a pipe. The ratio of these two effects is a dimensionless group known as the Reynolds number, Re , of any particular flow situation.

$$Re = d.u.\rho/\mu$$

where ρ/μ is the kinematic viscosity. Experiments carried out with a number of different fluids in straight pipes of different diameters have established that if the Reynolds number is calculated by making d equal to the pipe diameter (in this case the diameter of the test sections in the flow rig was 6 mm), and using the mean velocity, u , then, below a critical value of $Re = 2000$, flow will normally be laminar (viscous), any tendency to turbulence being damped out by viscous friction. In pipes, at values of Reynolds number exceeding 2000, flow will not necessarily be turbulent; however in straight pipes of constant diameter, flow can be assumed to be turbulent if the Reynolds number exceeds 4000 (Douglas et al, 1979). A graph of Reynolds number versus flow rate is given in the Appendix (page 205).

In the experimental rig flow rates in the range 0.2-4.4 l/min could be obtained. The range 0.2-0.7 was in the laminar regime, 0.7-1.0 was intermediate, and above 1.0 the flow is turbulent.

The shear stress at the pipe wall τ_w was calculated using the expression:

$$\tau_w = C_f.\rho.u^2/2$$

where C_f is the Fanning friction factor, taken from a plot of friction factor versus Reynolds number for smooth pipes (Coulson and Richardson, 1954, p334: the value for friction factor given in these charts is equal to $2C_f$). A plot of wall shear stress versus flow rate is given in the Appendix (page 197); the data from this study can then be compared with results of other workers who have expressed results in

terms of shear stress.

When a fluid flowing with a uniform velocity enters a pipe, a boundary layer forms at the walls and gradually thickens as the distance from the entry point increases. At a certain distance from the inlet, the boundary layers join at the axis of the pipe, and from that point onwards, occupy the whole cross-section and consequently do not increase in thickness. The flow is then fully developed. The medium in the experimental rig passed through a preliminary section of tubing 11 cm in length and 6 mm in diameter, before passing through the test sections, also of 6 mm diameter. At the start of the test sections, the flow may not have been fully developed due to the entrance effects which occur as a result of the fluid entering a pipeline of narrower diameter: the distance at which the flow becomes fully developed can be calculated using various empirical equations.

Coulson and Richardson (1954) suggest (as an approximate guide) that the inlet length, L_e , is given by:

$$L_e/d = 0.0288Re \quad \text{for } Re < 2500,$$

$$L_e/d = 50d \text{ to } 100d \quad \text{for } Re > 2500,$$

where d is the pipe diameter, i.e. 6 mm.

Thus the fluid flow may not be fully developed for a considerable distance (up to approximately 60 cm for the highest flow rates obtainable in the flow rig used in this work which is much longer than the preliminary section of 11 cm at the start of the test sections). However, analysis of the results shows that there was no difference between numbers of attached cells, or uptake per cell, for any of the sets of three test sections placed in series in each 'leg' of

the rig. This suggests that the entrance effects are minimal and that they can be disregarded.

2. Results and discussion

For each flow rate and glucose concentration used, the results for all the tubes were averaged, and the combined data from many experimental runs is shown in Figs. 4.2 to 4.8. Fig. 4.1 shows the type of results obtained from individual experiments. All of these graphs are based on results given in Table 8.1 in the Appendix (page 191). Data from individual experiments do not in general show statistically significant differences in glucose uptake over the flow rate range 1.0-4.4 l/min.

At each substrate concentration, the averaged results (see Figs 4.2 to 4.8), also suggest that increase in flow rate has no significant effect on the substrate uptake of the cells for the range of substrate concentrations tested (glucose from 0.02 g/l to 20.0 g/l).

Uptake is likely to be proportional to flow rate at relatively low flow rates and low substrate concentrations, when the supply of substrate to the biofilm is limited by mass transport considerations. At relatively high glucose concentrations/flow rates, saturation may be expected to occur. At the lowest glucose concentration shown (Fig 4.2) and flow rate (0.2 g/l; 0.2 l/min), the uptake was lower than the overall average uptake (ie average uptake for all flow rates). However, at this concentration there were considerable variations in the average for each flow rate, so the fact that the uptake is lower at the lowest flow rate may not be significant. As can be seen from Fig. 4.2, there was no general pattern of increased uptake with increased flow rate

at the lowest glucose concentration.

In some of the graphs showing average uptake versus flow rate at the various substrate concentrations (Figs 4.2 to 4.8), there appears to be a trough in the uptake for flow rates in the range of approximately 1 to 3 l/min. This is significant only at a low probability. However, as most of the graphs show averages of many individual results, this trend appears to be a 'real' phenomenon. For instance, the graph showing uptake at 2.0 g/l glucose over a half hour run (Fig 4.6) was prepared using the results from 105 individual tubes. There are no obvious explanations for this trend. It is possible that:-

(i) it could be due to a diffusional effect. It is possible that the diffusion layer around each cell may be thicker at these flow rates, and thus the rate of diffusion is limited. This would seem to be unlikely as flow rates greater than approximately 1 l/min the flow is turbulent, and the boundary layer thickness decreases as flow rate increases (see Figs 4.9 and 4.10: Fig 4.9 shows the flow velocities at distances up to 10 μm from the tube surface and Fig 4.10 shows the thickness of the laminar sublayer. The calculations for these graphs are shown in the Appendix on pages 206-208).

(ii) it may be due to a physiological effect on the bacteria of unknown cause. Flow rate has been shown to affect bacterial physiology in what may be a similar way, in at least one other case. Eighmy and Bishop (1985) showed that, following exposure to a shear stress of above 3.1 N/m^2 for a period of 10 minutes, the binding-protein-mediated aspartate transport system of a mixed biofilm (System 2) was inact-

ivated, and the membrane-bound aspartate proton system (System 1) was resolved. System 1 was described as a high-affinity, low-capacity proton symport, whereas System 2, which predominated at higher substrate concentrations, was described as a low affinity, high-capacity binding-protein-mediated transporter. Eighmy and Bishop concluded that the fluid downsweeps which are associated with turbulent flow in closed loop reactor systems, applied shear forces to the adhering cells and their extracellular glycocalyxes and caused the disruption of the binding-protein-mediated transport system by deforming the plastic outer membrane.

(iii) Another possible explanation for the negligible effect of changes in flow rate on the uptake of the cells relates the results obtained to the glucose uptake mechanisms observed in Pseudomonas (see the Introduction, page 29). The experiments in this study were carried out at a relatively low temperature, and it is therefore probable that a large proportion of the glucose was converted to gluconate and/or 2-ketogluconate (2-KG) outside the cell. Consequently it is difficult to draw firm conclusions about uptake since it is not known whether the substrate actively accumulated by the bacteria was glucose, gluconate or 2-ketogluconate or all three. The effective concentration of each at the cell surface could vary at different flow rates as a consequence of (i) the relative amount of glucose converted to the oxidation products and hence their relative concentration at the cell surface; (ii) the rate of loss of gluconate and 2-ketogluconate by diffusion away from the cell, and loss into the flowing medium, which would vary at different flow rates;

and (iii) the relative affinities of the bacteria for the three potential transport substrates.

At 4.4 l/min some anomalously high results were occasionally obtained. These have been indicated on the graphs with an asterisk. This is possibly due to the high degree of turbulence at these flow rates. Eddies may have broken through the boundary layers around the cells reducing the 'diffusion layer' and causing enhanced uptake.

3. Summary and Interpretation

The overall conclusion from these experiments is that fluid flow rate has little effect on the amount of glucose incorporated by the attached film of bacteria exposed to glucose concentrations in the range 0.2-20.0 g/l. This indicates that under conditions in which the concentration of glucose in the circulating fluid does not alter detectably (because the amount of medium in the rig is large, and hence the amount of glucose present is many orders greater than the amount the attached cells could assimilate), there is no physical influence of fluid flow rate on the ability of the bacteria to incorporate glucose.

This suggests that diffusion of glucose to the cell surface proceeds at a constant rate for all flow rates, which is improbable. If this were the case, glucose uptake would be a function only of glucose concentration, relative to the affinity of the bacterial uptake system(s) for glucose.

Alternatively, the results may be due to a physiological effect caused by increases in flow. A further investigation would be required to establish exactly how the bacterial physiology was affected.

4.1 Glucose uptake versus flow rate: results of
 individual experiments.

A: 2.0 g/l glucose

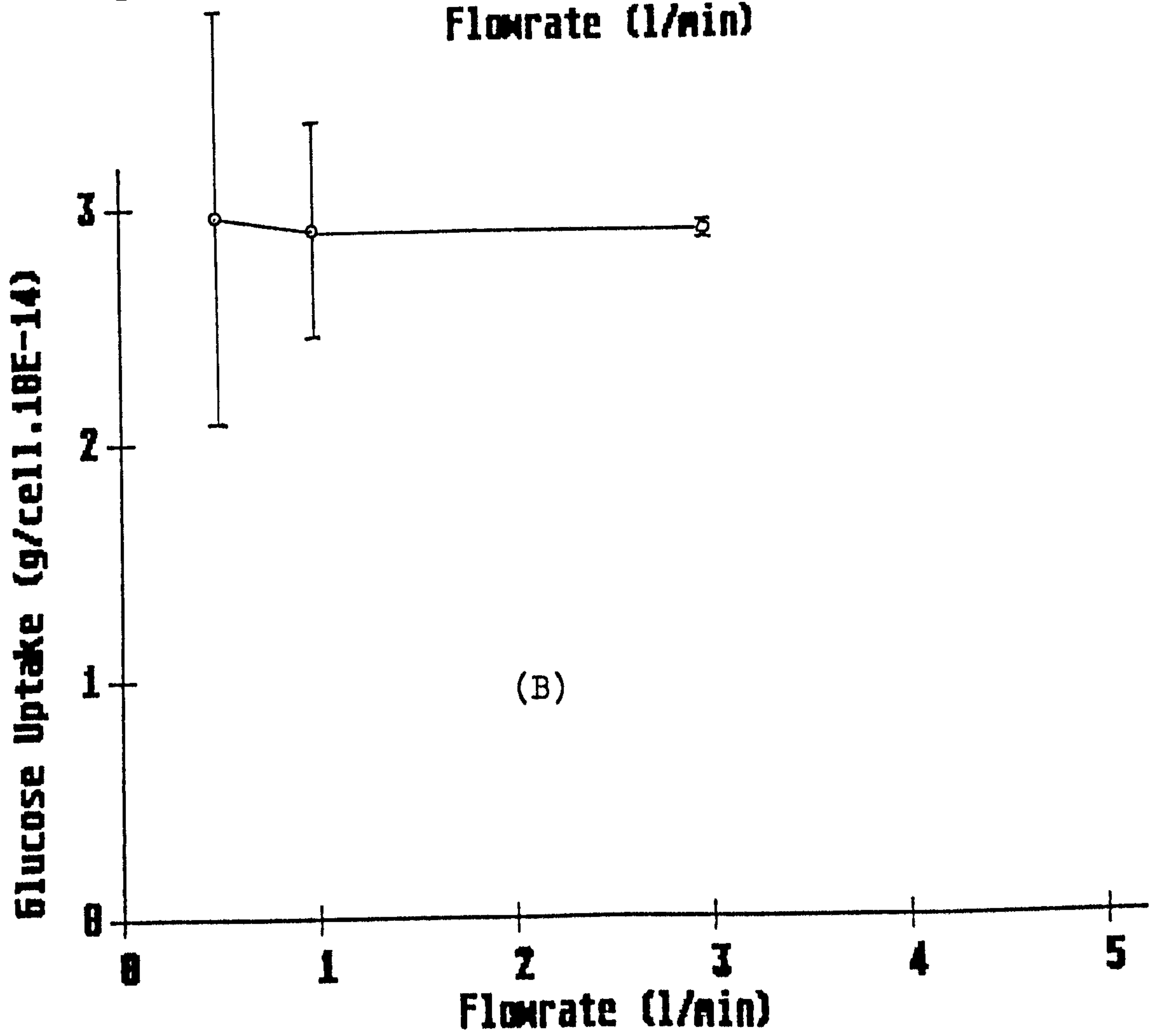
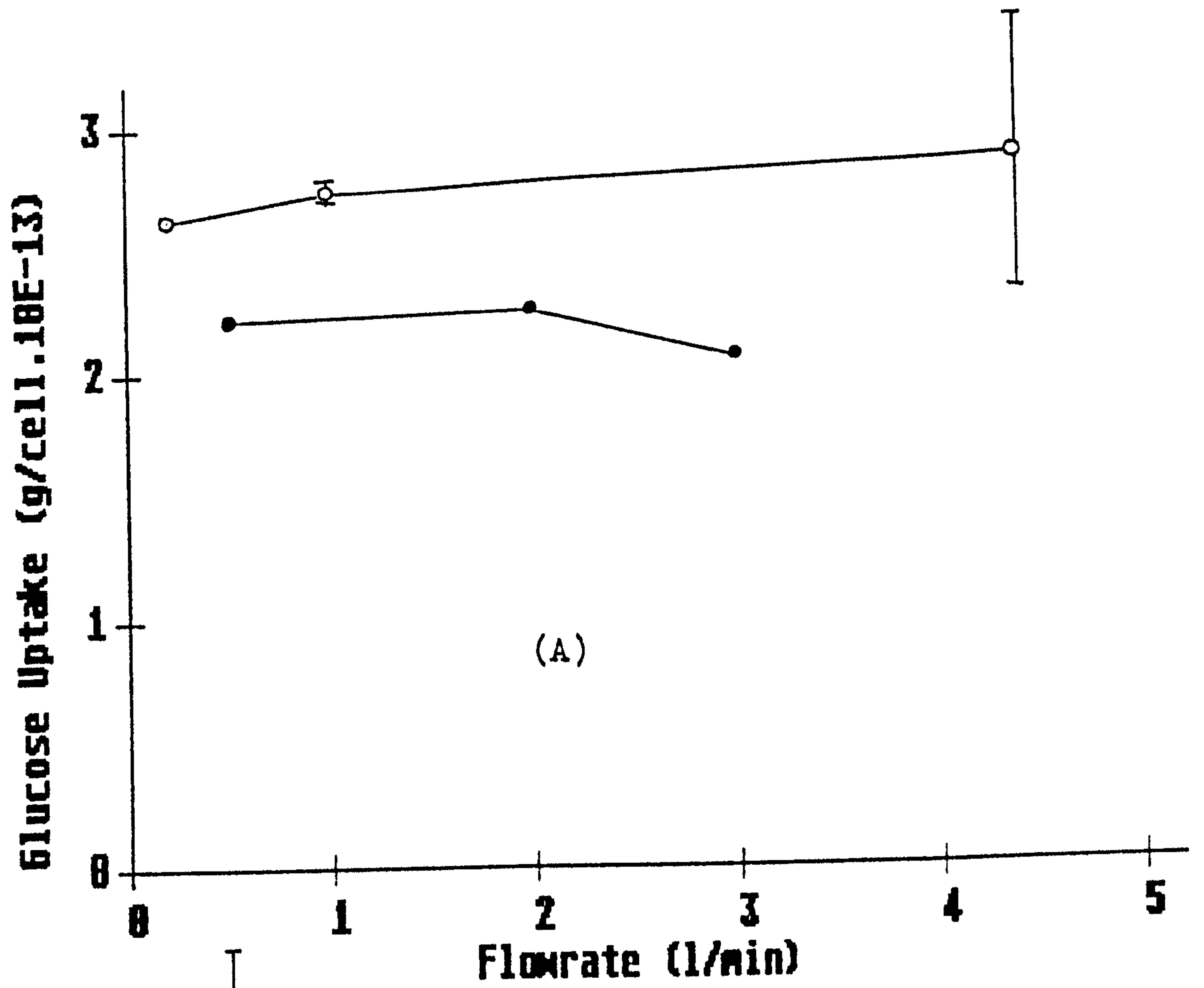
 Results of 16/1/87: 1 hour run period

 Results of 28/5/87: 30 minute run period

B: 0.2 g/l glucose

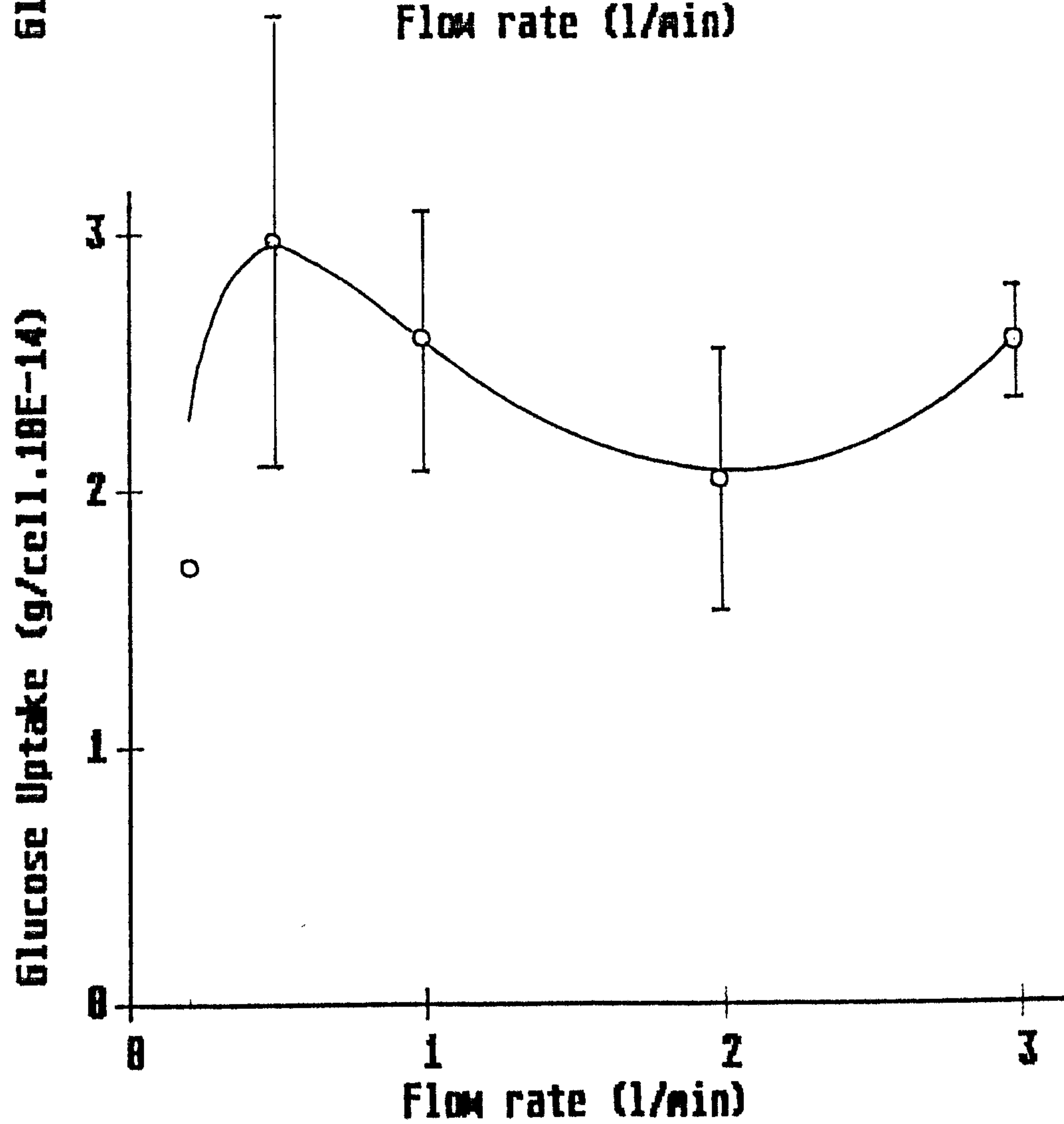
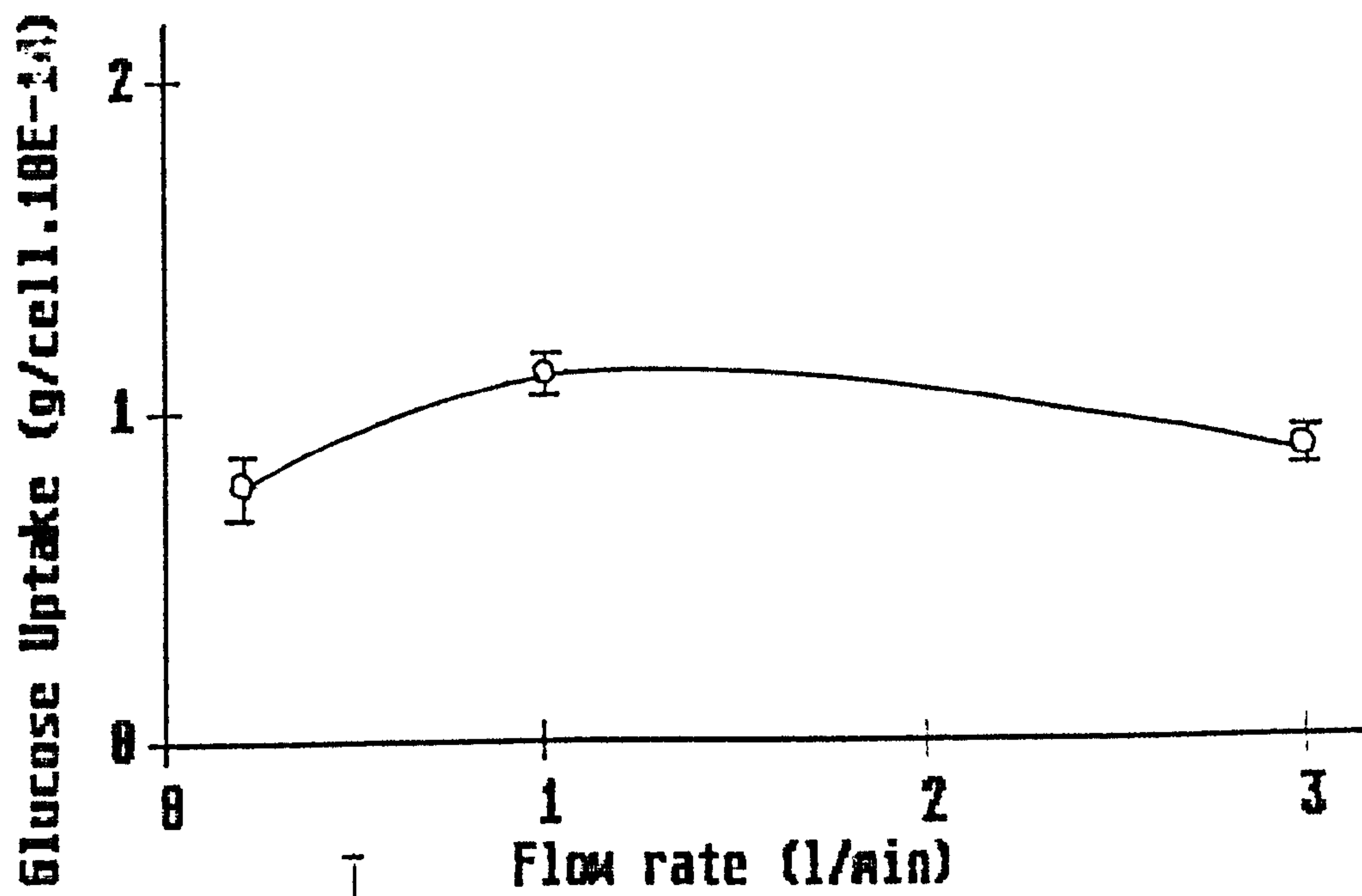
 Results of 8/1/86: 1 hour run period

Note: Where no error bar is shown, the point is derived
from a single result.



4.2 Glucose uptake versus flow rate during a
 30 min run for a glucose concentration of
 0.2 g/l.

4.3 Glucose uptake versus flow rate during a
 1 hour run for a glucose concentration of
 0.2 g/l.

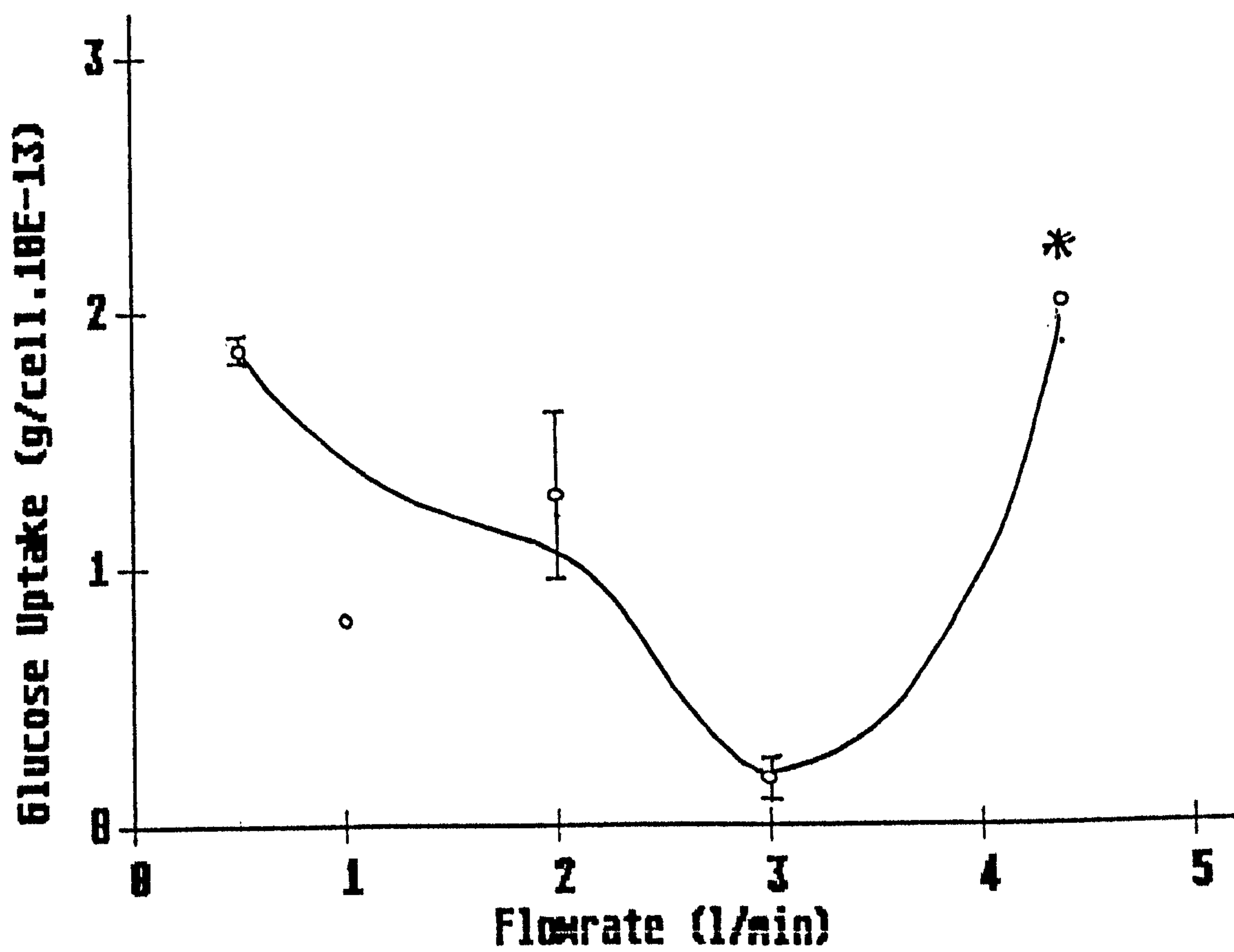
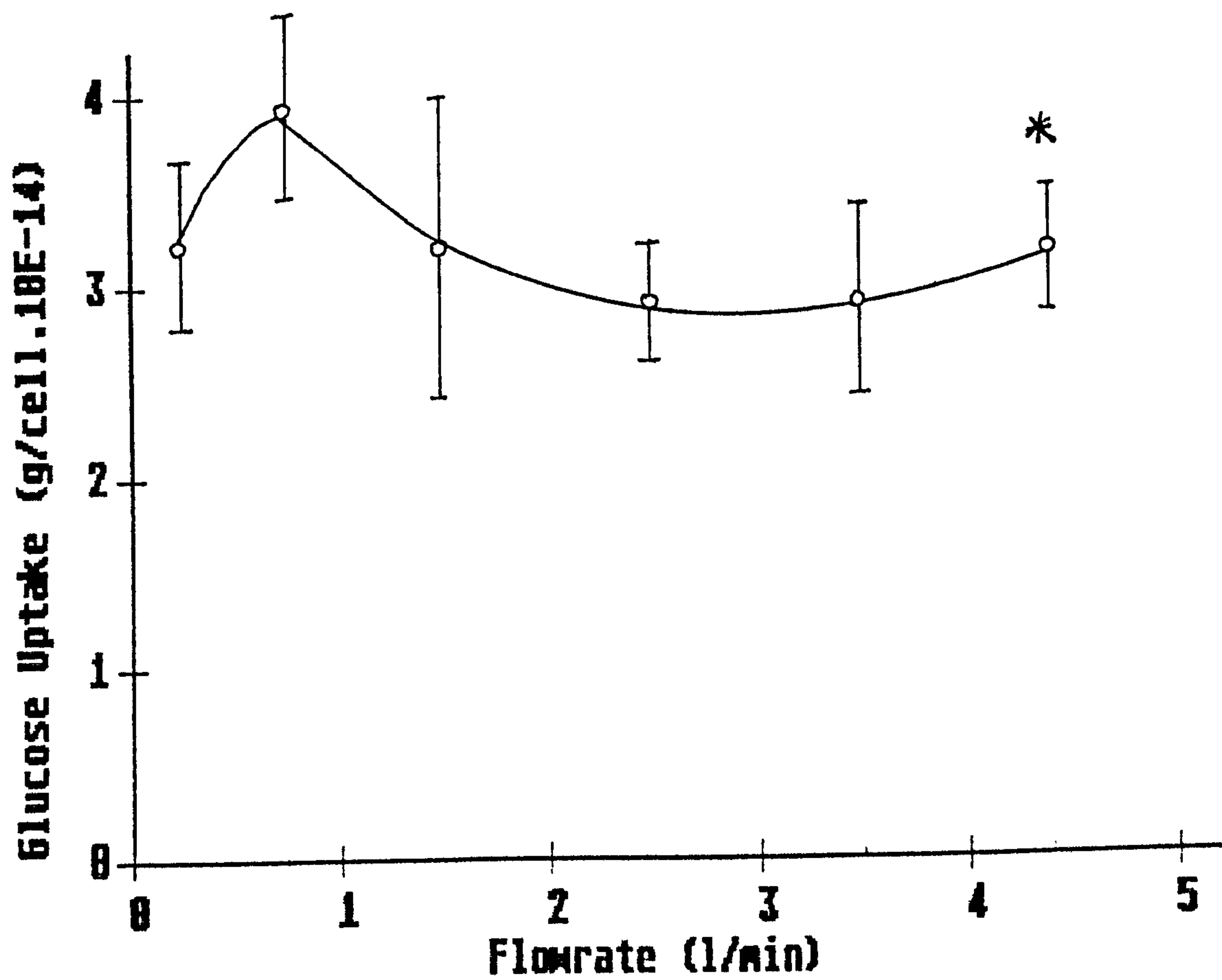


4.4 Glucose uptake versus flow rate during a
 30 min run for a glucose concentration of
 0.5 g/l.

4.5 Glucose uptake versus flow rate during a
 1 hour run for a glucose concentration of
 0.5 g/l.

Note: Where no error bar is shown, the point is derived
from a single result.

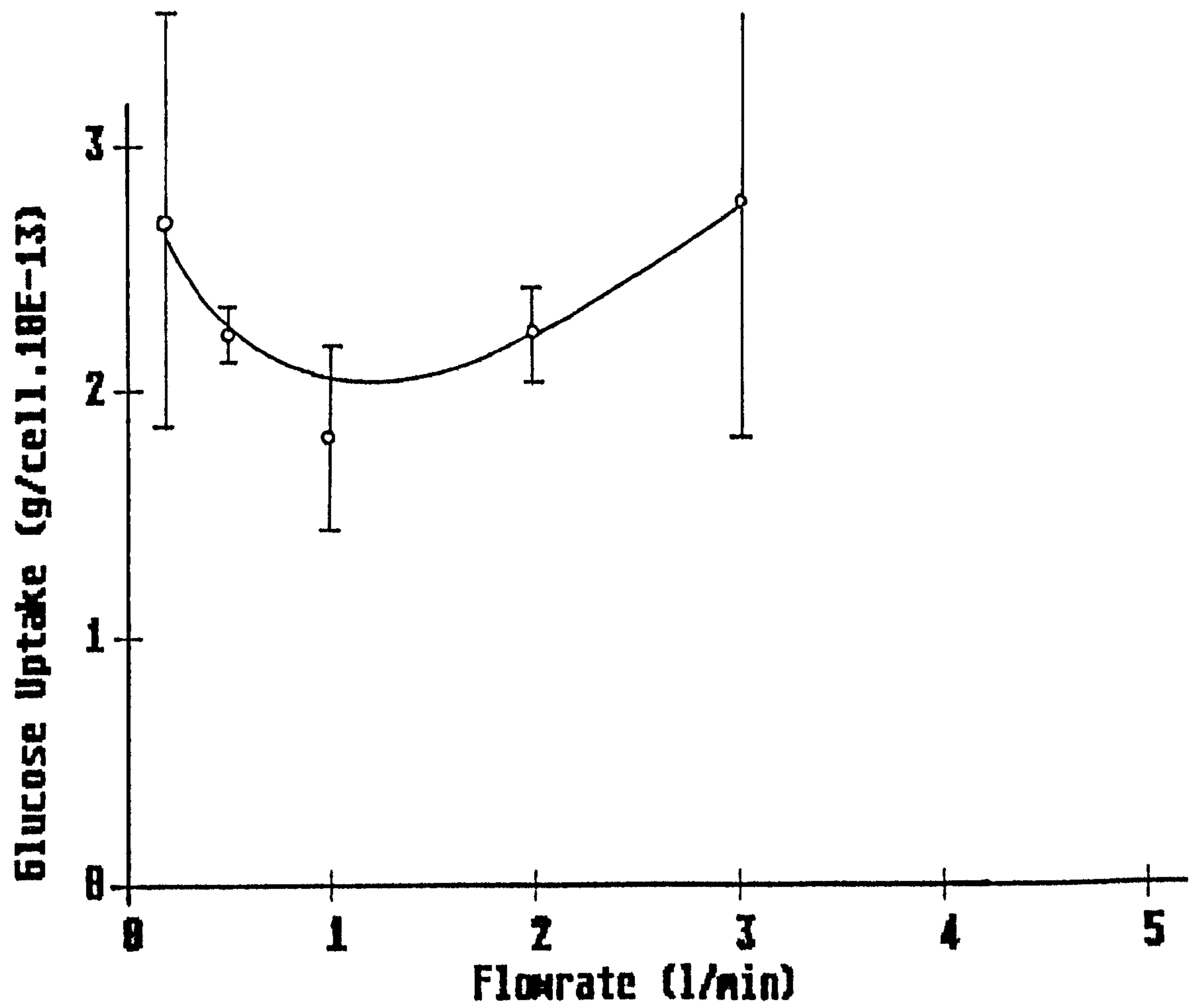
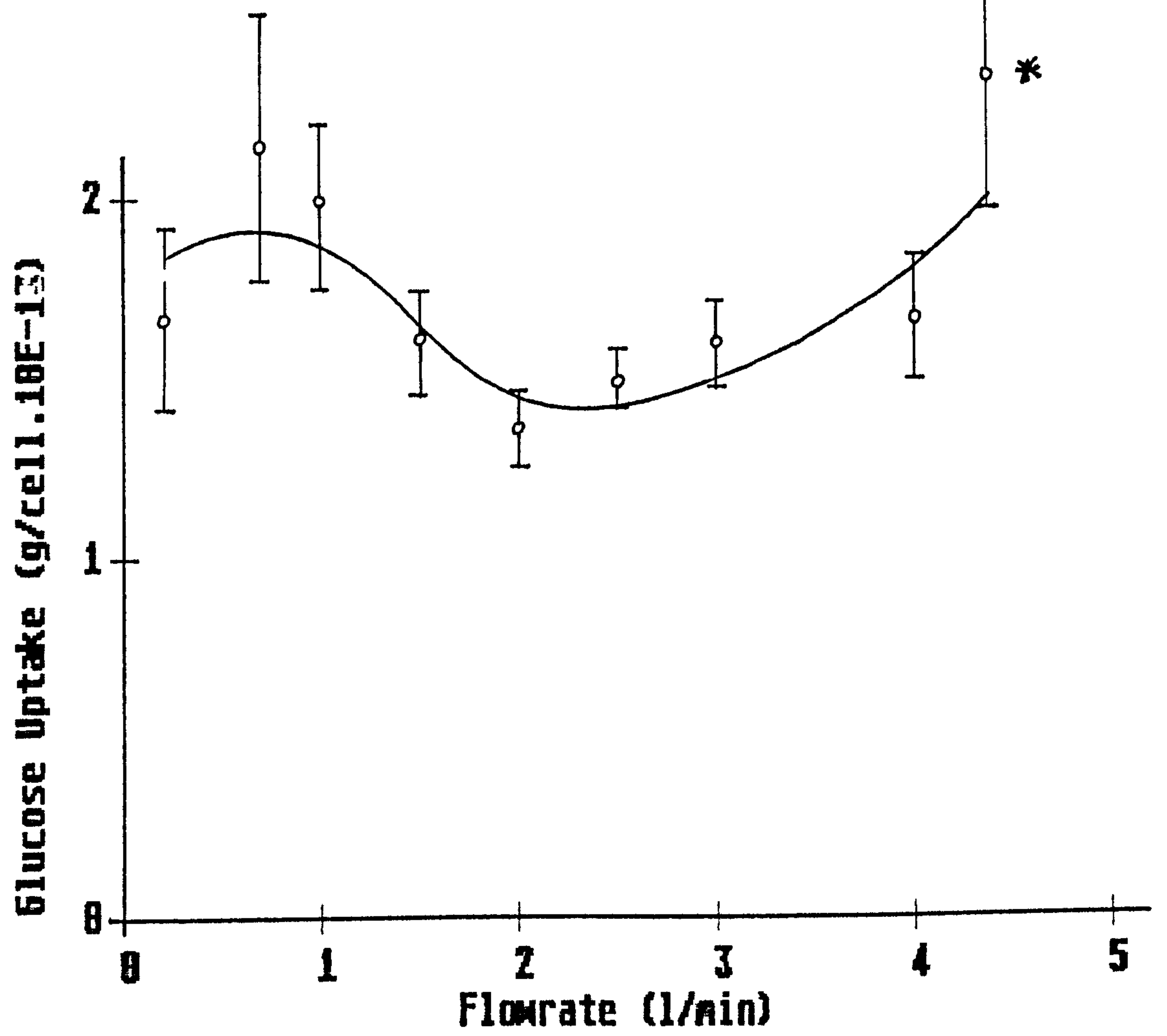
* Shows some anomolous high results were obtained at
4.4 l/min (not shown in data).



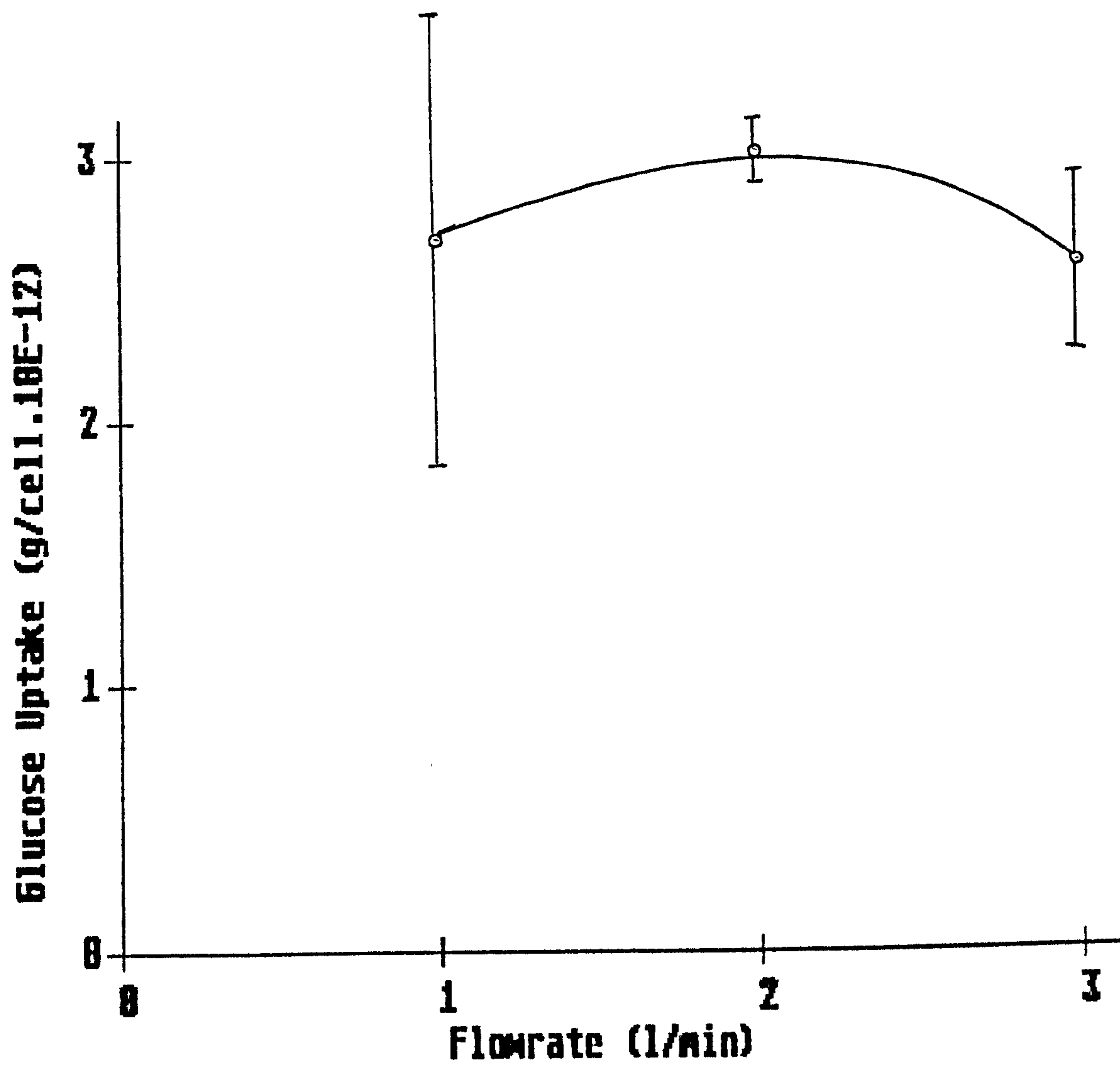
4.6 Glucose uptake versus flow rate during a
 30 min run for a glucose concentration of
 2.00 g/l.

4.7 Glucose uptake versus flow rate during a
 1 hour run for a glucose concentration of
 2.00 g/l.

* Shows some anomolous high results were obtained at
4.4 l/min (not shown in data).



4.8 Glucose uptake versus flow rate during a
 1 hour run for a glucose concentration of
 20.0 g/l.

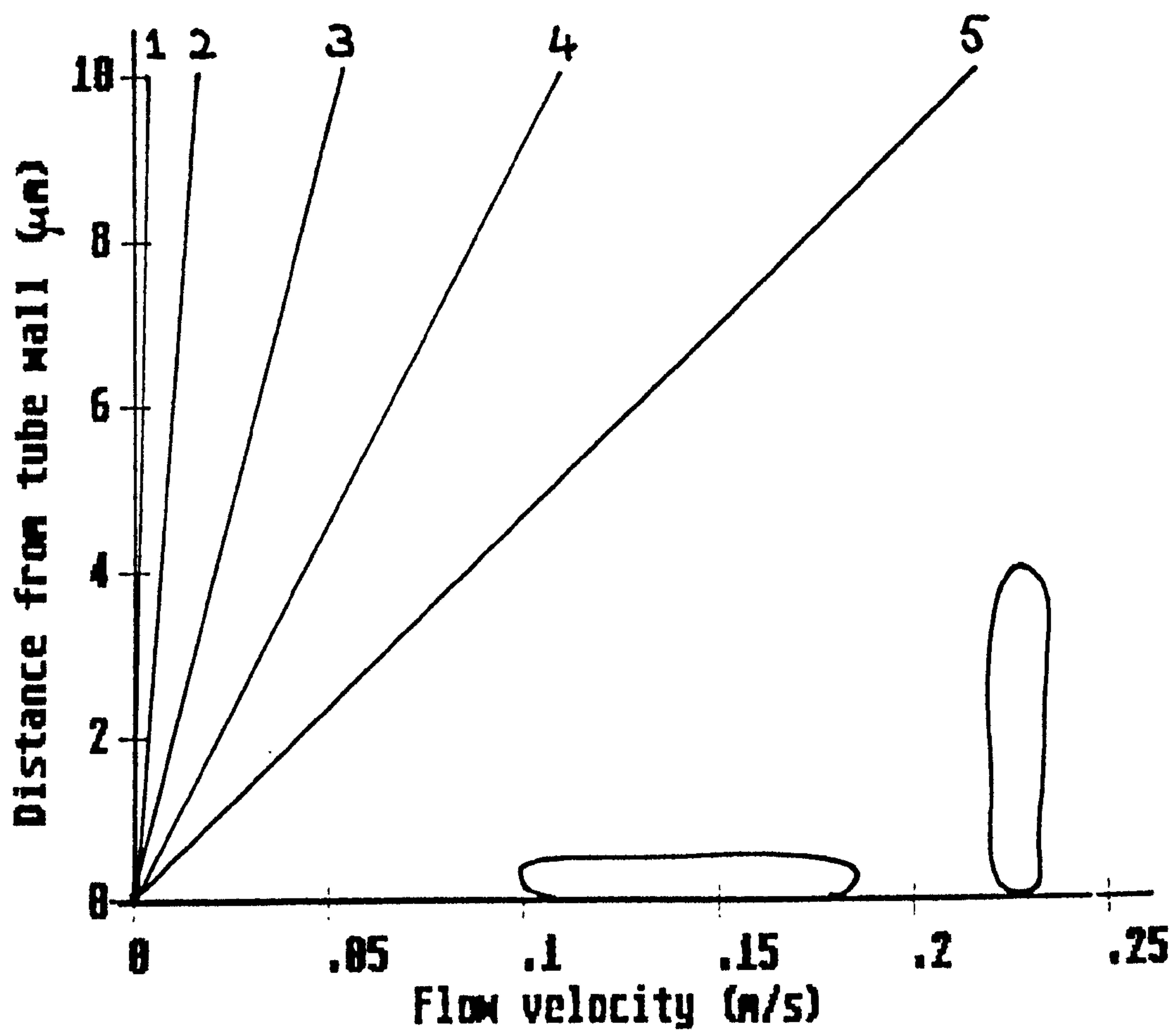


4.9 Velocity profiles for a distance of up to 10
 μm from the surface at different flow rates.

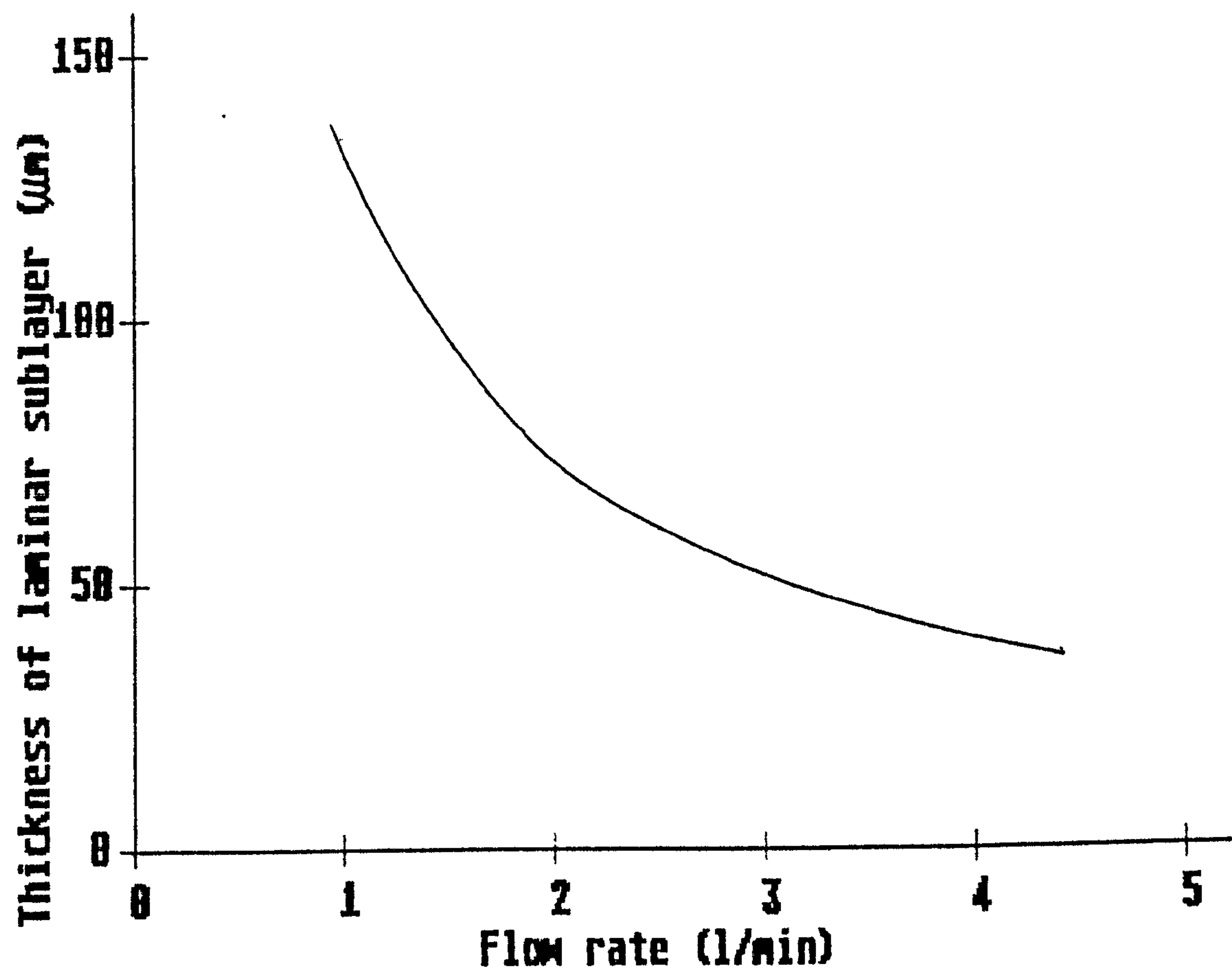
Key:

Line	Flow rate
1:	0.5 l/m
2:	1.0 l/min
3:	2.0 l/min
4:	3.0 l/min
5:	4.4 l/min

Relative size of bacteria are shown.



4.10 Thickness of laminar sublayer at different
 flow rates.



CHAPTER FIVE

EFFECT OF SUBSTRATE CONCENTRATION

1. Introduction

The glucose concentrations used in the experiments with the flow rig were in the range 0.02 g/l to 2.0 g/l (1.11×10^{-4} M to 1.11×10^{-2} M). The bacteria were able to grow in batch culture at these concentrations of glucose (see growth curves, p 199).

The bulk liquid could be assumed to be fully mixed if the Peclet number, Pe , is much greater than unity (Shieh, 1982). The Peclet number is defined as:

$$Pe = (\nu/D).Re$$

where ν is the kinematic viscosity of the medium, D is the diffusivity of the substrate, and Re is the Reynolds number. In these experiments, Pe was several orders of magnitude greater than one at the lowest value of Reynolds number obtained in the rig (see page 202). This indicates that convection rather than diffusion is predominant and the substrate concentration can be regarded as constant in the bulk liquid (Shieh, 1982).

A single particle, unaffected by the proximity of other particles, will be surrounded by a mass transfer boundary layer, which is the area inside an envelope which connects positions at 99% of the bulk fluid concentration, C_L . Its location in space depends both on the rate of reaction and mechanics of flow around the particle. When fluid velocity is increased the concentration immediately adjacent to the particle approaches the bulk liquid concentration (C_L) and

the concentration gradients are decreased. At high values of fluid velocity, the amount of substrate taken up per unit time becomes essentially constant, and the boundary layer is effectively of zero thickness. It is usual to model the physical situation by assuming that the mass-transfer boundary layer is of constant thickness (Atkinson, 1974).

Under steady state conditions the kinetics of the substrate uptake can be modelled by equating the diffusional transfer of reactants through the boundary layer with the consumption of the substrate by the biological particle. Thus:

$$R_p = h \cdot A_p \cdot (C_L - C^*) = N \cdot A_p,$$

where R_p is the mass of substrate converted per unit time, h is the mass-transfer coefficient, N represents the flux per unit area, and A_p is the surface area of a particle. If the reaction can be described by first order kinetics,

$$N = k_1 \cdot C^*,$$

where k_1 is a first-order rate coefficient. If this is substituted into the equation for R_p , C^* can be eliminated. This gives:

$$R_p = \left(\frac{1}{h} + \frac{1}{k_1} \right)^{-1} \cdot A_p \cdot C_L.$$

In the case of linear kinetics, the overall rate of reaction is linearly related to the bulk liquid concentration.

If the mass-transfer coefficient h is much greater than the kinetic coefficient k_1 , the reaction is determined by the value of the kinetic rate coefficient and is said to be reaction controlled; when k_1 is much greater than h liquid-

phase diffusion control exists. In the reaction controlled regime the interfacial concentration (C^*) approaches the bulk concentration, whereas with liquid-phase diffusion control the interfacial concentration falls to zero.

If both h and k_1 are of a similar magnitude, a significant diffusional resistance is involved, and in this situation the effect of increased fluid velocity is to increase the magnitude of the mass-transfer coefficient, which has the effect of decreasing the concentration gradients in the fluid and increasing the interfacial concentration. In some cases, for a small fluid velocity, a given physical situation may be diffusion controlled; high velocities may lead to reaction control and intermediate velocities to a condition of diffusion limitation.

2. Results and discussion

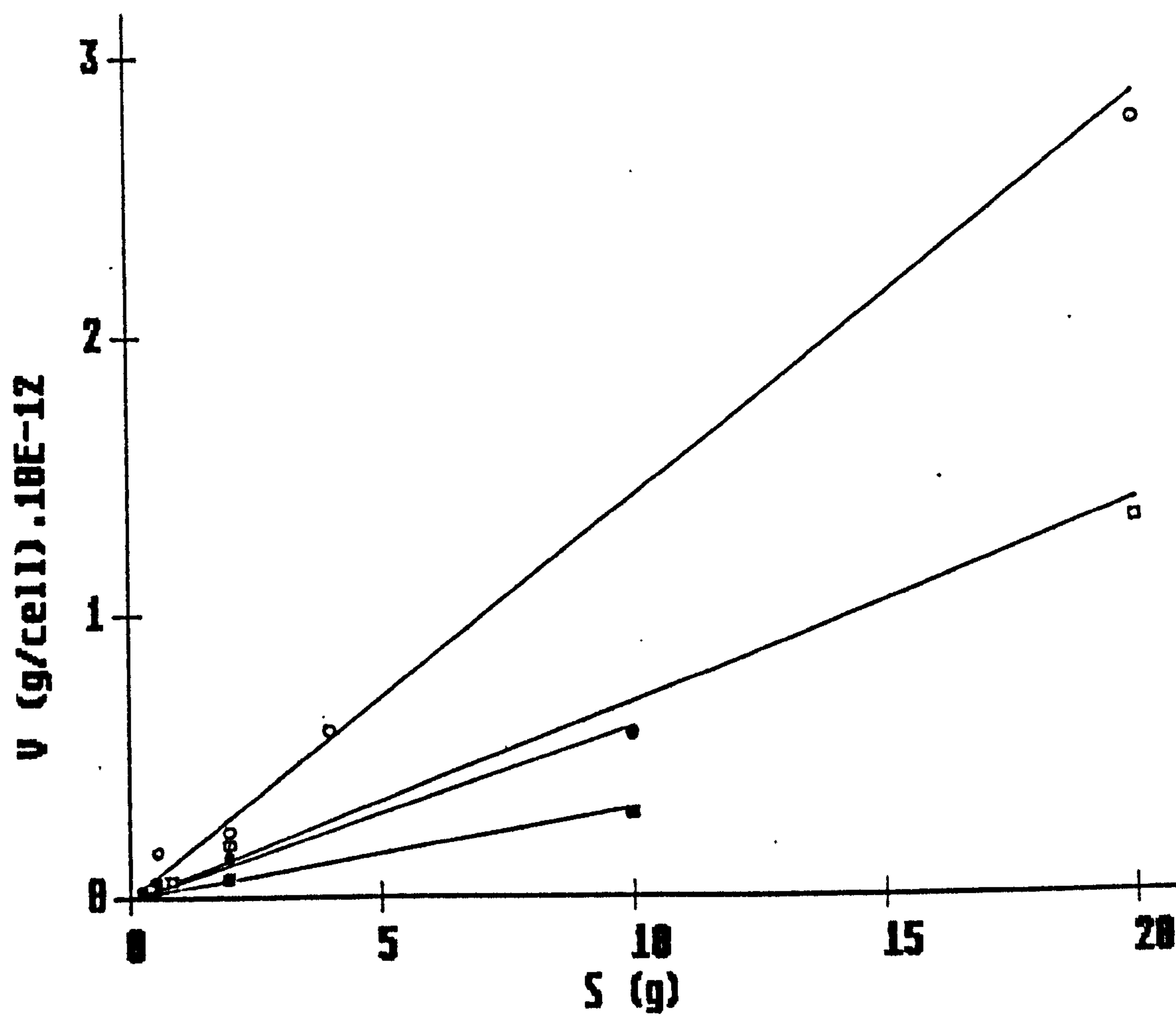
It has been shown in Chapter Four that flow rate has no significant influence on the glucose incorporation by biofilms in these experiments. Therefore the results for every tube exposed to a given substrate concentration in the rig were averaged, regardless of the flow rate which each tube experienced. The data on which these graphs are based is given in the Appendices (see pages 190-196). The results were plotted as a graph of uptake against substrate concentration (Fig. 5.1).

In addition the results were plotted as a Lineweaver-Burk graph (Fig. 5.2). This shows the reciprocal of the glucose uptake rate, $1/V$, versus the reciprocal of the glucose concentration supplied, $1/S$.

5.1 Average glucose uptake versus substrate concentration.

Key:

- Average uptake of attached cells in the flow rig over a period of 1 hour.
- Average uptake of freely suspended cells over a period of 1 hour.
- Average uptake of attached cells in the flow rig over a period of 30 minutes.
- Average uptake of freely suspended cells over a period of 30 minutes.



5.2 Lineweaver-Burk plot: showing the reciprocal of glucose incorporated per cell versus the reciprocal of glucose concentration.

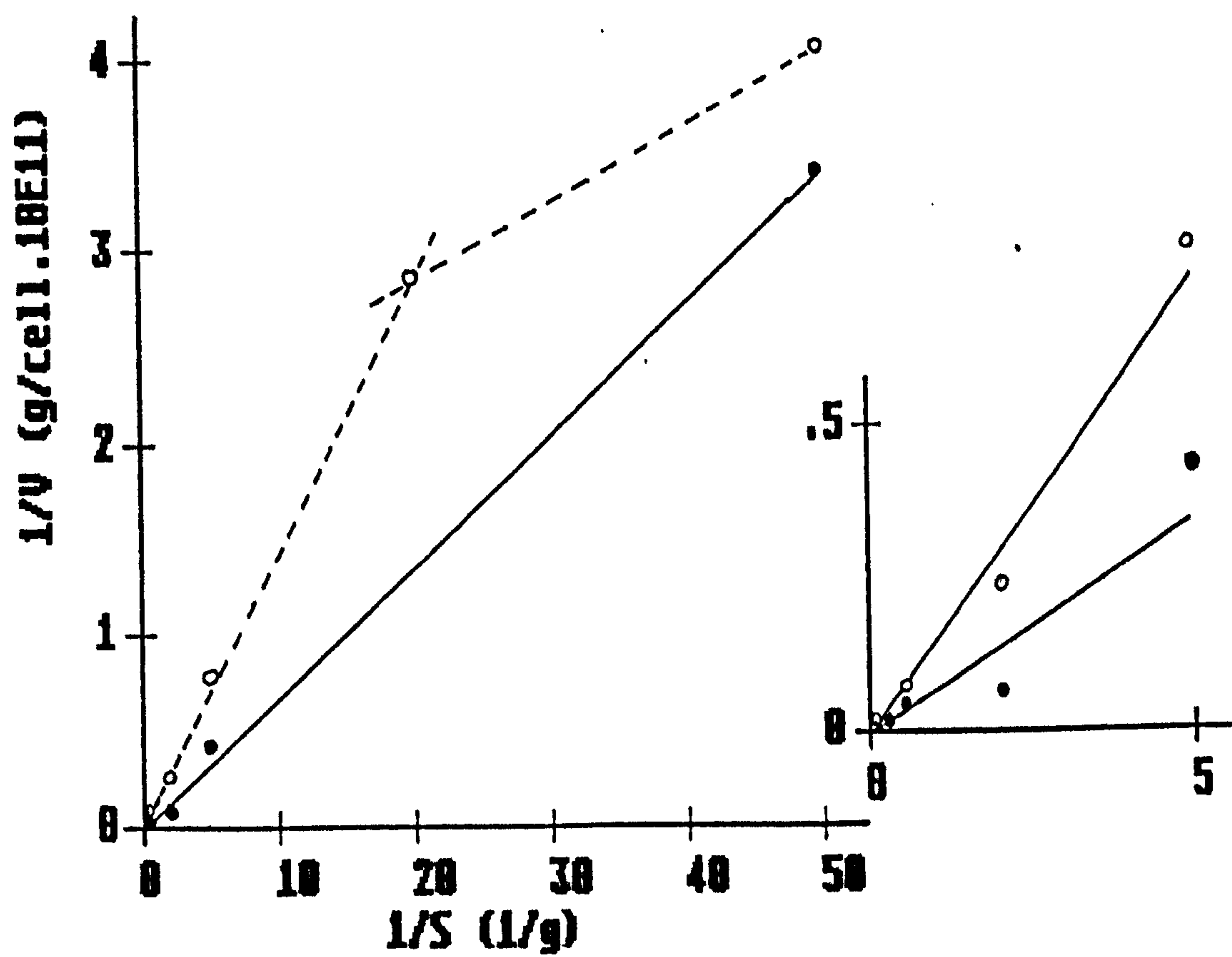
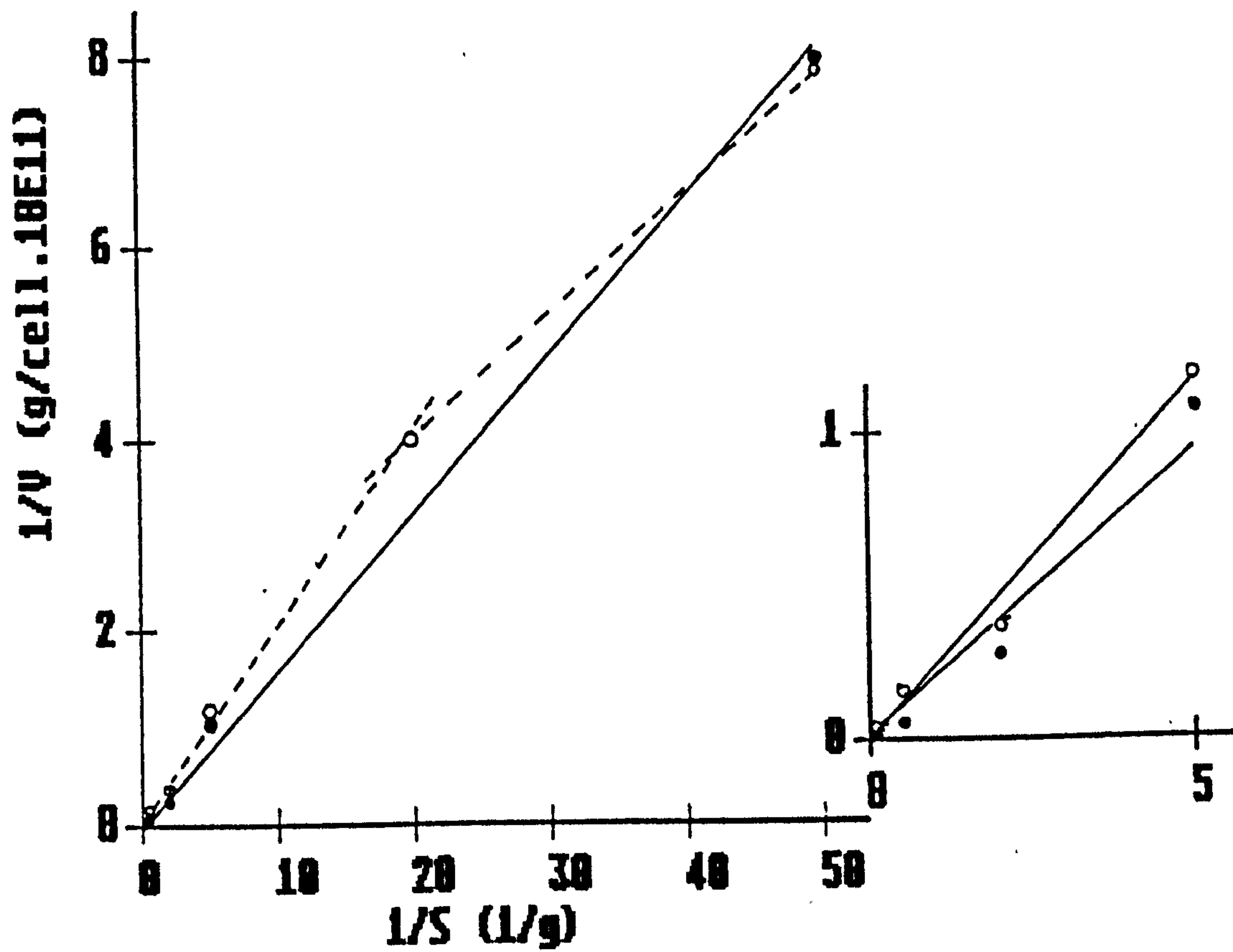
A: 30 minute period

- Freely suspended cells
- Attached cells

B: 1 hour period

- Freely suspended cells
- Attached cells

Inset graphs show detail close to origin.



The equation of the plot is:

$$\frac{1}{V} = \frac{K_m}{V_{max} S} + \frac{1}{V_{max}}$$

Further information on the Lineweaver-Burk plot is given in Aiba et al, 1973.

It can be seen from the graphs that glucose uptake is proportional to glucose concentration and to time, comparing the 0.5 and 1.0 hour samples. This confirmed that the time scale used in most experiments was realistic in falling in a period where linear kinetics prevailed. If biofilm growth had commenced an exponential increase in uptake would have been observed.

When the results were plotted as a Lineweaver-Burk graph the data could be extrapolated through the origin, indicating that saturation only occurred when the substrate concentration approached infinity: this implies the uptake was diffusion limited. The linear relationship between the quantity of glucose taken up and the concentration of glucose supplied was rather surprising, as there was no sign of saturation of the uptake rate even at 2% (w/v) glucose, and consequently no appearance of a rectangular hyperbolic relationship as would be expected if uptake were obeying Michaelis-Menten kinetics. If Michaelis-Menten kinetics were obeyed, the half-saturation constant (k_m) would be greater than, say, 1% (= 55mM). Thus this suggests that uptake was not limited by the activity of a high affinity glucose transport system. The results for attached bacteria in the flow rig can be compared to the uptake of freely suspended cells; a virtually identical Lineweaver-Burk plot obtained in this case.

A comparison of the rates of uptake by freely suspended cells and attached films showed the former to fall within the range seen for biofilms (Fig 5.1 and Tables 5.1-5.4). However the values were about half those calculated from a large number of biofilm experiments. This may reflect differences between attached and suspended populations, and could mean:-

(i) that the biofilms were made up mainly of the cells in the original suspension capable of rapid glucose uptake, which were able selectively to attach to the tube surfaces, leaving the less active cells in suspension;

(ii) that attachment to a surface could enable greater substrate capture (Several workers have suggested that attachment to a surface may affect the activity of bacterial cells, including Fletcher and Marshall, 1983; Savage, 1984);

(iii) that the metabolism of attached cells is affected by flow past the biofilm. It is possible, for instance, that cells subjected to high shear stresses may secrete more extracellular polymer. However the results obtained in this study do not seem to support this conjecture.

Caldwell and Lawrence (1986) used computer-enhanced microscopy to study the growth kinetics of bacterial microcolonies attached to the wall of a continuous-flow slide culture. They considered the growth rate of attached Pseudomonas fluorescens cells at 0.1 and 1.0 g/l glucose. They showed that 'at low substrate concentrations the growth rate varied depending on laminar flow velocity', and that growth was 'flow dependent' at a glucose concentration of 0.1 g/l and 'flow independent' at a concentration of 1.0 g/l,

indicating that the surface microenvironment became substrate depleted in the absence of sufficient laminar flow velocities. Their results show only the effect of allowing cells to grow in the absence of shear stress, and then increasing flow velocity to 0.1 m/s. They did not study the effect of varying flow rate past the surface, or directly measure glucose uptake of the cells, though cell growth was measured. It would be expected that attached cells would become substrate depleted over time in conditions of low substrate, and in the absence of a shear stress. Freely-suspended cells used in the present study were also shown to become substrate depleted in low glucose medium (see page 126 in Chapter 3).

The results from the present study appear to indicate that for flows in the range 0.2 - 4.4 l/m (with a constant nutrient concentration) substrate depletion does not occur. Although the initial uptake rates for freely suspended cells are approximately 50% of those for attached cells it is not certain that this is a significant difference.

Substrate uptake for biofilms exposed to different values of shear stress has been measured in very few studies. La Motta (1976) established a mixed biofilm in an annular reactor using a glucose medium. Although the flow dynamics were not well characterised, conditions were probably turbulent as the reactor was operated at high rotational speeds. He found that substrate uptake followed a zero-order rate law; the rate of film growth was found to be proportional to the initial substrate concentration in the reactor. One of the graphs shows that when the influent glucose concentration is 0.2 g/l (1.11×10^{-3} M) the intrinsic uptake

rate is 7.67×10^{-8} moles $\text{sec}^{-1} (\text{cm}^3 \text{film})^{-1}$. If the approximate number of cells per cubic centimetre of biofilm is estimated, the uptake obtained by La Motta can be compared with the results obtained at the same glucose concentration in this study. Assuming a cell volume of $1 \mu\text{m}^3$, with no spaces in the biofilm, the maximum cell density possible is 10^{12} cells/ cm^3 . The maximum cell volume of the Pseudomonas fluorescens cells used in this study was approximately $4 \mu\text{m}^3$: the maximum cell density possible is 2.5×10^{11} cells/ cm^3 . If a substantial volume of the biofilm is free space the cell density is likely to fall somewhere within the range 10^{11} to 10^{12} cells/ cm^3 . If these figures are used to approximate cell density, and units are converted to those used in the present study, the uptake obtained by La Motta was in the order of 5.0×10^{-14} to 5.0×10^{-13} g/cell.hour. The uptake obtained at the same glucose concentration in this research was 2.33×10^{-14} g/cell.hour. This is slightly below the range of La Motta, but is in the same order, which supports the validity of the results presented in this chapter. There were however many differences between the apparatus used by La Motta and the experimental apparatus used in this research. In addition, La Motta used a mixed biofilm, which was as much as $500 \mu\text{m}$ thick. In spite of the relative thickness of this film, and the poorly defined hydrodynamic conditions in his reactor, he states that 'reaction controlled' conditions prevail: i.e. the cells in the biofilm are not affected by diffusional resistances. This is not proved, and it seems likely that cells in the lower part of a biofilm several hundred microns thick would become substrate-limited under most circumstances.

3. Summary

Although surprising, the data seem unequivocally to show that the organisms in the thin biofilms used in my experiments took up glucose by a diffusion-rate-limited process. It is reasonable to presume that the transport process by which glucose enters the cells is of reasonably high affinity, with a K_m of the order 0.001 to 1 mM, which must mean that the effective concentration of glucose at the cell surface, exposed to the boundary layer, must be much lower than in the bulk fluid flowing in the system.

As suggested in Chapter 4, another possible explanation for the results obtained could be due to the pathways of glucose catabolism which are found in Pseudomonads (see page 29 in Chapter 1). It is possible that as shear stress was increased, the transport of glucose to the cell increased, but, simultaneously, the transport of gluconate/2-KG away from the cell also increased. If these two effects were balanced, then increases in flow rate would have little effect on the quantity of glucose incorporated into the cells. This may partially account for the fact that flow rates had virtually no effect on glucose uptake.

Averaged results showing uptake at various values of substrate concentration

Table 5.1: Uptake by attached cells during a period of 30 minutes.

Substrate concentration g/l	Uptake g/cell	Number of samples
0.02	1.26×10^{-15}	15
0.20	9.35×10^{-15}	9
0.50	3.36×10^{-14}	34
2.00	1.87×10^{-13}	102
20.00	1.30×10^{-12}	1

Table 5.2: Uptake by freely-suspended cells during a period of 30 minutes.

Substrate concentration g/l	Uptake g/cell	Number of repeats
0.02	1.28×10^{-15}	3
0.05	2.50×10^{-15}	3
0.20	8.50×10^{-15}	3
0.50	2.75×10^{-14}	3
2.00	7.00×10^{-14}	3
10.00	2.90×10^{-13}	3

Averaged results showing uptake at various values of substrate concentration

Table 5.3: Uptake by attached cells during a period of 60 minutes.

Substrate concentration g/l	Uptake g/cell	Number of samples
0.02	2.94×10^{-15}	8
0.20	2.33×10^{-14}	16
0.50	1.50×10^{-13}	14
2.00	2.35×10^{-13}	18
4.00	5.94×10^{-13}	3
20.00	2.76×10^{-12}	7

Table 5.4: Uptake by freely-suspended cells during a period of 60 minutes.

Substrate concentration g/l	Uptake g/cell	Number of samples
0.02	2.47×10^{-15}	3
0.05	3.48×10^{-15}	3
0.20	1.27×10^{-14}	3
0.50	4.18×10^{-14}	3
2.00	1.41×10^{-13}	3
10.00	5.77×10^{-13}	3

CHAPTER SIX

GENERAL CONCLUSIONS

1. Detachment studies

The results of the detachment studies were similar to those obtained by other workers. Duddridge et al, (1982) also considered detachment of Pseudomonas fluorescens from stainless steel surfaces. This was a detailed study in which the number of cells remaining on the surface was measured at many flow rates. Although the results of this study were expressed as numbers of cells remaining on the surface, and the percentage of cells remaining was not indicated, the trend of these results was similar to those obtained in this study, except that at high flow rates, there was a steeper fall in the numbers of cells which remained attached to the surface than was seen in the present study. However there was considerable variation between individual results, as was also seen in this study, and it is possible that the differences in the results are due to this.

2. Increase in uptake of attached cells with time

A similar pattern was found at all glucose concentrations tested. It was found that glucose uptake by attached cells increased approximately linearly up to an hour after the start of an experiment. After this there was little increase in uptake for a period. Glucose uptake then started to increase rapidly. It is not certain why this pattern of uptake was followed. It would appear that no similar studies of uptake have previously been carried out. Uptake has been observed in well-established biofilms, but generally over quite long time periods. It seems that this initial pattern

of substrate uptake has not previously been observed.

At the start of the experiment, the cells were grown up to late log phase. This means that during the attachment period, division of the cells may be occurring, and the cells attached to the tubes will therefore be small in size. After the tube was placed in the flow rig rapid uptake was observed: during this period the cells might have been growing. A period during which little glucose was taken up followed: the cells may have started to divide, and the subsequent rapid increase in uptake may have been due the greater number of cells present on the tubes.

This may explain the pattern of uptake which was observed. Although cell size was not measured quantitatively, observations showed that cells attached to tubes which had been in the flow rig for 0.5-1.0 hours often appeared larger than cells on control tubes which had not been placed in the rig after the attachment stage. It is possible that the procedure for attachment and subsequent incubation imposed a period of 'synchronous growth' on the attached population.

The uptake rates measured in these experiments were relatively high. The average dry weight of a single cell was 2.09×10^{-13} g, and at 0.5 and 2.0 g/l glucose, the average amount glucose taken up in the first 30 minutes was approximately equal to the dry weight of the cell.

One of the possible explanations for the high uptake rates (see page 150) is that cells may be producing large quantities of extracellular polysaccharides (EPS). Although this could be occurring, it does not seem to be very probable for the following reasons:

(i) large quantities of EPS were not observed by Pringle (1983) when attached cells (of this species) were selectively stained to show up EPS (this experiment was carried out under static conditions);

(ii) it was possible that production of EPS was stimulated by an increase in shear stress. However, there was no evidence that glucose uptake increased at higher shear stresses (see Chapter Four);

(iii) during the present study large quantities of EPS were never seen when attached cells which had been stained with acridine orange were observed at a magnification of x 1000.

The high uptake rates are conceivable if the cells were in the process of dividing. The rate of turnover of assimilated glucose may also be high. Uptake rates measured by La Motta (1976) for thicker mixed biofilms (see discussion on page 151) were in the same order as those measured in this study.

3. Effect of shear stress and substrate concentration

These conclusions are discussed in detail in Chapters Four and Five. It appears from this study as though shear stress has no effect upon uptake levels for the range of flow rates and nutrient concentrations used in this study. There was a direct linear relationship between glucose uptake and glucose concentration. A Lineweaver-Burk plot of the results suggests that the uptake is diffusion limited.

These results were surprising, as one would expect uptake of glucose to increase with flow rate. It is possible that the results were due to the rather complex, temperature

dependent pathways of glucose uptake which occur in Pseudomonas fluorescens. Lynch and Franklin (1977) have shown that at relatively low temperatures, Pseudomonas fluorescens cells convert a large proportion of glucose to gluconate and/or 2-ketogluconate extracellularly (see Introduction, page 31). At higher shear stresses the proportion of gluconate and 2-ketogluconate which diffused away from the cell might increase. If this was in approximate balance with the increased supply of glucose at higher flow rates, a net change of glucose uptake might be difficult to observe.

There are other possible, albeit less probable, explanations for the lack of significant increases in uptake at higher shear stress; these are discussed in detail in Chapters 4 and 5.

4. Comparison of uptake by attached cells and freely-suspended cells

In general, attached cells have been found to be more active than freely suspended cells. (see Introduction, page 28). The results of the present study showed that the rate of glucose uptake by attached cells was approximately 50% greater than that for freely suspended cells at a glucose concentration of 2.0 g/l; this may reflect differences between the attached and freely suspended populations (see the discussion of possible reasons for this on page 28). The differences were more pronounced at higher glucose concentrations.

5. Enumeration of attached cells

The number of cells attached to the tubes at the start of each run was measured by radiolabelling cells. At the end

of an experimental run, the number of original cells remaining on the surface could be measured. However, the extent of cell division during the run was not known: more information would have been obtained from the results if appropriate techniques for measurement of the total number of cells attached to each tube at the end of a run could have been devised. It would then have been possible to relate the observed pattern of uptake to the number of cells which were attached at any given time. In addition, the effect of flow rate on the growth of individual cells could have been established.

Because of the importance of determining the total number of cells attached at the end of the run, a considerable period of time was spent devising a technique for measuring this. The various approaches which were tested were discussed in Chapter Three. The method of eluting cells from the tubes using a surfactant and then measuring numbers of viable cells (by plate counts) was the most promising method. This method was used successfully to establish numbers of attached cells. The surfactant eluted a fixed percentage of the cells; possibly those which were not yet firmly attached. However, this technique was unsuccessful when used to measure numbers of cells on tubes which had been exposed to shear stress in the biofouling rig. This may have been because loosely attached cells had already been removed by exposing the biofilm to a shear stress. These findings indicate that the attachment process probably occurs in stages: it may take some time for cells to become firmly attached.

6. Study of monolayers rather than thick biofilms

Most of the biofilm studies which have been carried out previously have considered thick biofilms or undefined biofilms composed of many species of bacteria. In many cases, both conditions have applied (Bryers and Characklis, 1982; La Motta, 1976; Rittmann and McCarty, 1980b).

In the present study, the approach taken was to consider one of the simplest situations possible. The biofilms were monolayers, composed of a single species. They were pre-attached, so the medium in the rig contained few bacteria (only those which detached from the test sections). Time periods were short, and the volume of medium in the flow rig was large in comparison to the size of the test sections, so concentration of substrate in the medium could be regarded as constant.

In spite of the relative simplicity of the system, the results obtained were not easy to explain. This highlights the difficulties of interpreting the results from the more complex systems which are frequently studied. Many of the statements made in the literature are simplistic, and assumptions are often made which have no rigorous foundation. For instance, La Motta (1976) states that cells in biofilms which he investigated were not affected by diffusional resistances, even though these biofilms were 500 μm thick. For the majority of species, the maximum dimension of a cell lies within the range 1-10 μm (Singleton and Sainsbury, 1981). It is therefore likely that the microenvironment surrounding cells deeper within a 500 μm biofilm would be very different from that experienced by cells nearer to the surface.

If monolayers are used, the cells in a biofilm are exposed to uniform conditions, and the interpretation of the

results is less likely to be confounded by hidden variables.

7. Limitations of the Biofouling Rig

The main problem with the apparatus used in this study was the difficulty in finding a technique to measure numbers of cells present on the tubes at the end of an experiment. It is probable that a suitable technique could be devised eventually, but it was not possible in the time period available for this study. Cutting up the stainless steel test sections in order to actually observe the attached biofilm was very difficult, and this was a major problem.

Another disadvantage was the fact that flow rates below 0.2 l/min could not be obtained. It was also difficult to obtain test sections of materials other than stainless steel which were of a suitable size for the flow rig.

The rig was originally constructed to hold three 15 cm test sections. This was changed so that three 5 cm tubes could be fitted in each 'leg' of the rig, so that 9 test sections could be used. This was a limitation: for example, in the study of the change of glucose uptake with time, three flow rates and three time periods could be investigated in any one experiment, but there were no replicate tubes for each condition.

8. Improvement of the flow rig

The test sections used by Pedersen (1982a), described on page 22, had many advantages. He developed a system in which fluid flowed through banks of slides which were held in a special carrier.

The biofouling rig used in the present study would have

been rather more flexible if it had been constructed to hold test sections similar to those developed by Pedersen. The system could be built to hold banks of slides in series, which would increase the number of readings obtainable in each experiment. Carriers could be constructed to hold test sections of different materials, and determination of the number of cells on each surface at the end of an experiment would be easier. If transparent surfaces were used, a rapid and simple technique such as the crystal violet method described on page 36 could be utilised.

If this type of test section were incorporated into a flow rig with similar features to the one used in the present study, a highly flexible system would result. The system would be further improved if it were adapted to take flow meters which would cover flow rates below 0.2 l/min.

With such a system, information could be obtained more rapidly, and a wider variety of parameters tested. It seems particularly important to study the effect of reducing flow rates below 0.2 l/min, in order to check whether uptake at a given substrate concentration is reduced.

9. Possible future studies

Some of the results found in the present study could not be explained conclusively. A sensible starting point for a future study might be an investigation of some of the effects observed in the present study. The most important objective would be to evaluate the effect on the results of the uptake systems involved with glucose catabolism in Pseudomonas fluorescens. There would be two obvious starting points:

(1) The simplest test would be to carry out uptake experi-

ments similar to those described in this study at a higher temperature: at 30°C most glucose is catabolized via the direct uptake and phosphorylation of glucose (Lynch and Franklin, 1978). This need not be a lengthy study; experiments at only one glucose concentration would be necessary. If there were significant differences between the results obtained at 15°C and 30°C, analysis of the data might throw more light on the results obtained in the present study. Selective stains could also be used to check whether the production of extracellular polysaccharides is increased at higher flow rates.

(ii) A different bacterial species (with a simpler glucose uptake system) could be used for further uptake studies. The effect of flow rate on these biofilms could be investigated, and again, comparison of the results with those from the present study might provide valuable information.

If these two key investigations were carried out, it is likely that useful facts would emerge. Information on the effect of flow rate on biofilms in other situations would have been acquired, and the effect of the uptake systems found in Pseudomonas fluorescens on the results of the present study would be clearer.

There are many other experiments which could be carried out using the biofouling rig which would yield valuable results. Decisions on which experiments would be most appropriate could only be made after an analysis of the results of the experiments suggested in paragraphs (i) and (ii) above. If changes in shear stress were found to affect uptake by the biofilms at 30°C, or the biofilms of a different species,

this should be investigated further. It might then be possible to model the results in order to assess whether changes in mass transport of substrate to the cells, or changes in the physiological activity of the cells (or both), were responsible for the effect of flow rate on uptake.

A further line of investigation, of considerable interest, would be to compare the results of replicate uptake experiments carried out using monolayers and thicker biofilms. No similar experiments appear to have been reported; it would be useful to know to what extent uptake rates vary as biofilm thickness is increased from a monolayer of cells.

There are many other studies which could be carried out using the biofouling rigs which would be likely to yield results which would further current understanding. Experiments using different microorganisms and substrates are possible. A vast quantity of data could be gained very quickly if the modifications to the biofouling rig suggested on page 165 were implemented. Studies of attachment to different surfaces could then be carried out if desired.

Interesting and useful results have been obtained in this study from experiments using the biofouling rig, which should provide a foundation for further research. However, there are still many avenues which have yet to be explored.

CHAPTER SEVEN

REFERENCES AND SELECTED BIBLIOGRAPHY

- ABSOLOM, D. R., LAMBERTI, F. V., POLICOVA, Z., ZINGG, W., van OSS, C. J. and NEUMANN, A. W. 1983. Surface thermodynamics of bacterial adhesion. *Appl. Environ. Microbiol.* 46: 90-97.
- AFTRING, R. P., and TAYLOR, B. F. 1979. Assessment of microbial fouling in an ocean thermal energy conversion experiment. *Appl. Environ. Microbiol.* 38: 734-739.
- AIBA, S., HUMPHREY, A. B., and MILLIS, N. F. 1973. *Biochemical Engineering*. 2nd Ed. Academic Press, Inc., New York and London.
- ALBRIGHT, L. J., McCABE, S. K., and MAY, B. E. 1986. Attached and free floating bacterioplankton in Howe Sound, British Columbia, a coastal marine fjord-embayment. *Appl. Environ. Microbiol.* 51: 614-621.
- ALLDREDGE, A. L. and COHEN Y. 1987. Can microscale chemical patches persist in the sea? Microelectrode study of marine snow, fecal pellets. *Science* 235: 689-691.
- ARCURI, E. J., DONALDSON, T. L. 1981. Biofilm kinetics. *Biotechnol. Bioeng.* 23: 2149-2149.
- ATKINSON, B. 1974. *Biochemical Reactors*. Pion Limited, London.
- ATKINSON, B., and FOWLER, H. W. 1974. The significance of microbial film in fermenters. *Adv. Biochem. Eng.* 3: 221-227.
- BAIER, R. E., 1980. Substrata influences on adhesion of microorganisms and their resultant new surface properties.

- In: Adsorption of Microorganisms to Surfaces, pp 59-104.
Edited by G. Bitton & K. C. Marshall. Wiley, New York.
- BAILEY, J. E. and OLLIS, D. F. 1977. Biochemical engineering fundamentals. McGraw-Hill, Inc.
- BELAS, M. R. and COLWELL, R. R. 1982. Adsorption kinetics of laterally and polarly flagellated Vibrio. J. Bacteriol. 151: 1568-1580.
- BENEFIELD, L., and MOLZ, F. 1985. Mathematical simulation of a biofilm process. Biotechnol. Bioeng. 27: 921-931.
- BERGER, L. R., and BERGER, J. A. 1986. Countermeasures to microbiofouling in simulated ocean thermal energy conversion heat exchangers with surface and deep ocean waters in Hawaii. Appl. Environ. Microbiol. 51: 1186-1198.
- BERGSTROM, I., HEINÄNEN, A., and SALONEN, K. 1986. Comparison of acridine orange, acriflavine and bisbenzimidazole stains for enumeration of bacteria in clear and humic waters. Appl. Environ. Microbiol. 51: 664-667.
- BLANCH, H. W., and DUNN, I. J. 1974. Modelling and simulation in biochemical engineering. Adv. Biochem. Eng. 3: 127-165.
- BONGRAND, P., CAPO, C., and DEPIEDS, R. 1982. Physics of cell adhesion. Progress in Surface Science. 12: 217-286.
- BRANFORD WHITE, C. J., ROWE, P. S., HORSMAN, M. R. and SPICER, A. B. 1977a. Characteristics of glucose uptake and cell wall envelope composition in L-Forms of Pseudomonas aeruginosa. Microbios Letters. 5: 67-70.
- BRANFORD WHITE, C. J., HORSMAN, M. R., and ROWE, P. S. 1977b. Glucose uptake of Pseudomonas aeruginosa and its carbenicillin-induced L-form. Biochem. Soc. Trans. 571st Meeting, Dublin.

- BRATBAK, G. 1985. Bacterial biovolume and biomass estimations. Appl. Environ. Microbiol. 49: 1488-1493.
- BRIGHT, J. J. 1983. PhD Thesis, University of Warwick.
- BRIGHT, J. J., and FLETCHER, M. M. 1983a. Amino acid assimilation and electron transport system activity in attached and free-living marine bacteria. Appl. Environ. Microbiol. 45: 818-825.
- BRIGHT, J. J., and FLETCHER, M. M. 1983b. Amino acid assimilation and respiration by attached and free-living populations of a marine pseudomonas sp.. Microb. Ecol. 9: 215-226.
- BROCK, T. D. 1967. Bacterial growth rate in the sea: direct analysis by thymidine autoradiography. Science 155: 81-83.
- BROWN, C. M., ELLWOOD, D. C., and HUNTER, J. R. 1977. Growth of bacteria at surfaces: influence of nutrient limitation. FEMS Microbiol. Lett. 1: 163-166.
- BRUNK, C. F., JONES, K. C., and JAMES, T. W. 1979. Assay for nanogram quantities of DNA in cellular homogenates. Anal. Biochem. 92: 497-500.
- BRYERS, J. D., and CHARACKLIS, W. G. 1981. Early fouling biofilm formation in a turbulent flow system: overall kinetics. Water Res. 15: 483-491.
- BRYERS, J. D., and CHARACKLIS, W. G. 1982. Processes governing primary biofilm formation. Biotechnol. Bioeng. 24: 2451-2476.
- BUSSCHER, H. J., UYEN, M. H. W. J. C., VAN PELT, A. W. J., WEERKAMP, A. H., and ARENDS, J., 1986. Kinetics of adhesion of the oral bacterium Streptococcus sanguis CH3 to polymers of different surface free energies. Appl. Environ. Microbiol. 51: 910-914.

- BUTTON, D. K. 1985. Kinetics of nutrient limited transport and microbial growth. *Microbiological Reviews* 49: 290-297.
- CALDWELL, D. E., BRANNAN, D. K., MORRIS, M. E., and BETLACH, M. R. 1981. Quantitation of microbial growth on surfaces. *Microb. Ecol.* 7: 1-11.
- CALDWELL, D. E., and LAWRENCE, D. E. 1986. Growth kinetics of Pseudomonas fluorescens microcolonies within the hydrodynamic boundary layers of surface microenvironments. *Microb. Ecol.* 12: 299-312.
- CESARONE, C. P., BOLOGNESI, C., and SANTI, L. 1979. Improved microfluorimetric DNA determination in biological material using Hoechst 33258. *Anal. Biochem.* 100: 188-197.
- CHRISTENSEN, B. E., KJOSBAKKEN, J., and SMIDSRØD, O. 1985. Partial chemical and physical characterisation of two extracellular polysaccharides produced by marine, periphytic Pseudomonas sp. strain NCMB 2021. *Appl. Environ. Microbiol.* 50: 837-845.
- CHARACKLIS, W. G., and COOKSEY, K. E. 1983. Biofilms and microbial fouling. *Advances in Applied Microbiology.* 29: 93-138.
- CHARACKLIS, W. G., TRULEAR, M. G., BRYERS, J. D., and ZELVER, N. 1982. Dynamics of biofilm processes: methods. *Water Res.* 16: 1207-1216.
- CLARK, J. R., MESSENGER, D. I., DICKSON, K. L., and CAIRNS, J. 1978. Extraction of ATP from Aufwuchs communities. *Limnol. Oceanogr.* 23: 1055-1059.
- CLARKE, K. R., and JOINT, I. R. 1986. Methodology for estimating numbers of free-living and attached bacteria in estuarine water. *Appl. Environ. Microbiol.* 51: 1110-1120.

- CORPE, W. A. 1970. An acid polysaccharide produced by a primary film-forming bacterium. *Dev. Ind. Microbiol.* 2: 402-412.
- CORPE, W. A. 1979. Microbial surface components involved in adsorption of microorganisms onto surfaces. In: *Adsorption of Microorganisms to Surfaces*, pp. 106-139. Edited by G. Bitton & K. C. Marshall. Wiley, New York.
- COSTERTON, J. W. 1980. Some techniques involved in study of adsorption of microorganisms to surfaces. In: *Adsorption of Microorganisms to Surfaces*, pp 403-423. Edited by G. Bitton and K. C. Marshall. Wiley, New York.
- COSTERTON, J. W., GEESEY, G. G., and CHENG, K.-J. 1978. How bacteria stick. *Sci. Am.* 238: 86-95.
- COSTERTON, J. W., and IRWIN, R. T., and CHENG, K.-J. 1981. The bacterial glyco-calyx in nature and disease. *Ann. Rev. Microbiol.* 35: 299-324.
- COSTERTON, J. W., MARRIE, T. J. and CHENG, K.-J. 1985. Phenomena of bacterial adhesion. In: *Bacterial Adhesion to Surfaces*, pp 3-43. Edited by D. C. Savage and M. M. Fletcher. Plenum Publishing Corp., New York.
- COULSON, J. M., and RICHARDSON, J. F. 1954. *Chemical Engineering*. Vol. 1. Pergamon Press Ltd., London.
- CROUCH, C. F., FOWLER, H. W. and SPIER, R. E. 1985. Adhesion of animal cells to surfaces: the measurement of critical surface shear stress permitting attachment or causing detachment. *J. Chem. Tech. Biotechnol.* 35B: 273-281.
- CUSSLER, E. L. 1984. *Diffusion*. Cambridge University Press.
- DALEY, R. J., and HOBBIE, J. E. 1975. Direct counts of aquatic bacteria by a modified epifluorescence technique. *Limnol. Oceanogr.* 20: 875-882.

- DANIELS, S. L. 1980. Mechanisms involved in sorption of microorganisms to solid surfaces. In: Adsorption of Microorganisms to Surfaces, pp 7-58. Edited by G. Bitton and K. C. Marshall. Wiley-Interscience.
- DEFLAUN, M. F., PAUL, J. H., and DAVIS, D. 1986. Simplified method for dissolved DNA determination in aquatic environments. Appl. Environ. Microbiol. 52: 654-659.
- DERJAGUIN , B. V., and LANDAU, L. 1941. Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. Acta Physicocim. URSS. 14: 633-662.
- DEXTER, S. C., SULLIVAN, J. D., WILLIAMS J., and WATSON, S. W. 1975. Influence of substrate wettability on the attachment of marine bacteria to various surfaces. Applied Microbiology. 30: 298-308.
- DOROSZEWSKI, J., GOLAB-MEYER, Z., and GURYN, W. 1979. Adhesion of cells in flowing suspensions: effects of shearing force and cell kinetic energy. Microvascular Research 18: 421-433.
- DOUGLAS, J. F., GASIOREK, J. M., and SWAFFIELD, J. A. 1979. Fluid Mechanics. Pitman Publishing Limited, London.
- DROSTE, R. L., and KENNEDY K. J. 1986. Sequential substrate utilisation and effectiveness factor in fixed biofilms. Biotechnol. Bioeng. 28: 1713-1720.
- DUDDRIDGE, J. E., KENT, C. A., and LAWS J. F. 1982. Effect of surface shear stress on attachment of Pseudomonas fluorescens to stainless steel under defined flow conditions. Biotechnol. Bioeng. 24: 153-164.
- EAGON, R. G., and PHIBBS JR., P. V. 1971. Kinetics of transport of glucose, fructose, and mannitol by

- Pseudomonas aeruginosa. Can. J. Biochem. 49: 1031-1041.
- EGAN, B. 1987. Marine microbial adhesion and its consequences. In: Microbes in the sea, pp 220-228. Edited by M.A. Sleight. Ellis Horwood Ltd., Chichester.
- EIGHMY, T. T., and BISHOP, P. L. 1985. The effect of turbulence on the binding-protein-mediated aspartate transport system in thin wastewater biofilms. Appl. Environ. Microbiol. 50: 120-124.
- EISENBERG, R. C., BUTTERS, S. J., QUAY, S. C. and FREIDMAN, S. B. 1974. Glucose uptake and phosphorylation in Pseudomonas fluorescens. J. Bacteriol. 120: 147-153.
- ELBEIN, A. D., SANFORD, B. A., RAMSAY, M. A. and PAN, Y. T. 1981. Effect of inhibitors on glycoprotein biosynthesis and bacterial adhesion. In: Adhesion and Microorganism Pathogenicity, pp 270-287. Pitman Medical, Tunbridge Wells.
- ERIKSON, L. C. et al. 1980. Measurement of DNA damage in unlabelled mamalian cells analysed by alkaline elution and a fluorimetric DNA assay. Anal. Biochem. 106: 169-174.
- FLETCHER, M. 1976. The effects of proteins on bacterial attachment to polystyrene. J. Gen. Microbiol. 94: 400-404.
- FLETCHER, M. 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. 23: 1-6.
- FLETCHER, M. 1979a. A microautoradiographic study of the activity of attached and free-living bacteria. Arch. Microbiol. 122: 271-274.
- FLETCHER, M. 1979b. The attachment of bacteria to surfaces in aquatic environments. In: Adhesion of Microorganisms to

- Surfaces, pp 87-108. Edited by D. C. Ellwood, J. Melling, P. Rutter. Academic Press.
- FLETCHER, M. 1980a. Adherence of marine microorganisms to smooth surfaces. In: Bacterial Adherence, pp 345-374. Edited by E. H. Beachey. Chapman and Hall.
- FLETCHER, M. 1980b. The question of passive versus active attachment mechanisms in nonspecific bacterial adhesion. In: Microbial Adhesion to Surfaces, pp 197-210. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent. Ellis Horwood, Chichester.
- FLETCHER, M. 1983. The effects of methanol, ethanol, propanol and butanol on bacterial attachment to surfaces. J. Gen. Microbiol. 123: 633-641.
- FLETCHER, M. 1984. Comparative physiology of attached and free-living bacteria. In: Microbial Adhesion and Aggregation, pp 223-232. Edited by K. C. Marshall. Dahlem Konferenzen 1984. Springer-Verlag. Berlin, New York, Heidelberg, Tokyo.
- FLETCHER, M. 1985. The effect of solid surfaces on the activity of attached bacteria. In: Bacterial Adhesion to Surfaces, pp 339-362. Edited by D. C. Savage and M. Fletcher. Plenum Publishing Corp., New York.
- FLETCHER, M. 1986. Measurement of glucose utilization by Pseudomonas fluorescens that are free-living and that are attached to surfaces. Appl. Environ. Microbiol. 52: 672-676.
- FLETCHER, M. 1987. How do bacteria attach to solid surfaces? Microbiol. Sciences 4: 133-136.
- FLETCHER, M., and FLOODGATE, G. D. 1973. An electron-microscopic demonstration of an acidic polysaccharide

- involved in the adhesion of a marine bacterium to solid surfaces. *J. Gen. Microbiol.* 74: 325-334.
- FLETCHER, M., and LOEB, G. I. 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. *Appl. Environ. Microbiol.* 37: 67-72.
- FLETCHER, M., and LATHAM, M. J., LYNCH, J. M., and RUTTER, P. R. 1980. The characteristics of interfaces and their role in microbial attachment. In: *Microbial Adhesion to Surfaces*, pp 67-78. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent. Horwood, Chichester.
- FLETCHER, M., and MARSHALL, K. C. 1982. A bubble contact method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. *Appl. Environ. Microbiol.* 44: 184-192.
- FLETCHER, M., and MARSHALL, K. C. 1983. Are solid surfaces of ecological significance to aquatic bacteria? In: *Advances in Microbial Ecology*, vol. 6, pp 199-236. Edited by K. C. Marshall. Plenum Publishing Corp., New York.
- FLETCHER, M., and PRINGLE, J. H. 1985. Effect of surface free energy and medium surface tension on bacterial attachment to solid surfaces. *J. Colloid and Interfaces* 104: 5-14.
- FOWLER, H. W., and MCKAY, A. J. 1980. The measurement of microbial adhesion. In: *Microbial Adhesion to Surfaces*, pp 143-161. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent. Ellis Horwood, Chichester.
- FUHRMAN, J. A., and AZAM, F. 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in

- marine surface waters: evaluation and field results. Mar. Biol. 66: 109-120.
- GAUDY JR, A. F., OBAYASHI, A., and GAUDI, E. T. 1971. Control of growth rate by initial substrate concentration at values below maximum rate. Appl. Microbiol. 22: 1041-1047.
- GEESEY, G. G., and MORITA, R. Y. 1981. Relationship of cell envelope stability to substrate capture in a marine psychrophilic bacterium. Appl. Environ. Microbiol. 42: 533-540.
- GORDON, A. S. 1987. Influence of alginate on attachment of Vibrio spp. to stainless steel surfaces in seawater. Appl. Environ. Microbiol. 53: 1175-1177.
- HARRIS, N. P., and HANSFORD, G. S. 1976. A study of substrate removal in a microbial film reactor. Water Res. 10: 935-943.
- HEATH, L. S., SLOAN, L. S., and HEATH, H. E. 1986. A simple and generally applicable procedure for releasing DNA from bacterial cells. Appl. Environ. Microbiol. 51: 1138-1140.
- HERSON, D. S., MCGONIGLE, B., PAYER, M. A., and BAKER, K. H. 1987. Attachment as a factor in the protection of Enterobacter cloacae from chlorination. Appl. Environ. Microbiol. 53: 1178-1180.
- HEUKELEKIAN, H., AND HELLER, A. 1940. Relation between food concentration and surface for bacterial growth, J. Bacteriol. 40: 547-558.
- HOBBIE, J. E., DALEY, R. J., and JASPER, S. 1977. A method for counting bacteria on Nuclepore filters. Appl. Environ. Microbiol. 33: 1225-1228.
- HODSON, R. E., MACCUBBIN, A. E., and POMEROY, L. R. 1981. Dissolved adenosine triphosphate utilization by free-

- living and attached bacterioplankton. Mar. Biol. 64: 43-51.
- HOLLIBAUGH, J. T., FUHRMAN, J. A. and AZAM, F. 1980. Radioactive labeling of natural assemblages of bacterioplankton for use in trophic studies. Limnol. Oceanogr. 25: 172-181.
- HORROCKS, D. L. 1977. The H number concept, technical report 1095 NUC 77-IT, Scientific Instruments Division, Beckman Instruments.
- HSIEH, K. M., LION, L. W., and SHULER, M. L. 1985. Bioreactor for the study of defined interactions of toxic metals and biofilms. Appl. Environ. Microbiol. 50: 1155-1161.
- HUNT, J. C. and PHIBBS JR., P. V. 1983. Regulation of alternate peripheral pathways of glucose catabolism during aerobic and anaerobic growth of Pseudomonas aeruginosa. J. Bacteriol. 154: 793-802.
- JAMES, D. W., SUSLOW, T. V., and STEINBACK, K. E. 1985. Relationship between rapid, firm adhesion and long-term colonization of roots by bacteria. Appl. Environ. Microbiol. 50: 392-397.
- JEFFREY, W. H., and PAUL, J. H. 1986a. Activity of an attached and free-living Vibrio sp as measured by thymidine incorporation, p-indonitrazolium reduction, and ATP/DNA ratios. Appl. Environ. Microbiol. 51: 150-156.
- JEFFREY, W. H., and PAUL, J. H. 1986b. Activity measurements of planktonic microbial and microfouling communities in a eutrophic estuary. Appl. Environ. Microbiol. 51: 157-162.
- KANG, K. S., VEEDER, G. T., MIRRASOUL, P. J., KANEKO, T., and COTTRELL, I. W. 1982. Agar-like polysaccharide produced by

- a Pseudomonas species: production and basic properties. Appl. Environ. Microbiol. 43: 1086-1091.
- KAPUSCINSKI, J., and SKOCZLAS, B. 1977. Simple and rapid fluorimetric method for DNA microassay. Anal. Biochem. 83: 252-257.
- KARL, D. M. 1979. Measurement of microbial activity and growth in the ocean by rates of stable ribonucleic acid synthesis. Appl. Environ. Microbiol. 38: 850-860.
- KARL, D. M. 1981. Simultaneous rates of ribonucleic acid and deoxyribonucleic acid synthesis for estimating growth and cell division of aquatic microbial communities. Appl. Environ. Microbiol. 42: 802-810.
- KARL, D. M., WINN, C. D., and WONG, D. C. L. 1981a. RNA synthesis as a measure of microbial growth in aquatic environments. I. Evaluation, verification and optimization of methods. Marine Biol. 64: 1-12.
- KARL, D. M., WINN, C. D., and WONG, D. C. L. 1981b. RNA synthesis as a measure of microbial growth in aquatic environments. II. Field applications. Mar. Biol. 64: 13-21.
- KARL, D. M., and BOSSARD, P. 1985. Measurement and significance of ATP and adenine nucleotide pool turnover in microbial cells and environmental samples. J. Microbiol. Methods 3: 125-139.
- KIDD-HAACK, S., BITTON G., and LAABES, D. 1985. Epiphytic bacteria: development of a method for determining respiring bacteria on leaves. J. Appl. Bacteriol. 59: 545-548.
- KIRCHMAN, D. 1983. The production of bacteria attached to particles suspended in a freshwater pond. Limnol. Oceanogr. 28: 858-872.

- KIRCHMAN, D., NEWELL, S. Y., and HODSON, R. E. 1986. Incorporation versus biosynthesis of leucine: implications for measuring rates of protein synthesis and biomass production by bacteria in marine systems. *Mar. Ecol. Prog. Ser.* 32: 47-59.
- KIRKPATRICK, J. P., McINTIRE, L. V., and CHARACKLIS, W. G. 1980. Mass and heat transfer in a circular tube with biofouling. *Water Res.* 14: 117-127.
- KJELLEBERG, S., HUMPHREY, B. A., and MARSHALL, K. C. 1982. Effect of interfaces on small starved marine bacteria. *Appl. environ Microbiol.* 43: 1166-1172.
- KJELLEBERG, S., HUMPHREY, B. A., and MARSHALL, K. C. 1983. Initial phases of starvation and activity of bacteria at surfaces. *Appl. Environ. Microbiol.* 46: 978-984.
- KOCH, A. L., and WANG, C. H. 1982. How close to the theoretical diffusion limit do bacterial uptake systems function? *Arch. Microbiol.* 131: 36-42.
- LABARCA, C., and PAIGEN, K. 1980. A rapid, simple, and sensitive DNA assay procedure. *Anal. Biochem.* 102: 344-352.
- LADD, T. I., COSTERTON, J. W., and GEESEY, G. G. 1979. Determination of the heterotrophic activity of epilithic microbial populations. In: *Native Aquatic Bacteria: Enumeration, Activity and Ecology*, pp 180-185. Edited by J. W. Costerton and R. R. Colwell. ASTM, Philadelphia.
- LA MOTTA, E. J. 1976. Kinetics of growth and substrate uptake in a biological film system. *Appl. Environ. Microbiol.* 31: 286-293.
- LAVIE, S., and STOTZKY, G. 1986. Adhesion of the clay minerals montmorillonite, kaolinite, and attapulgite

- reduces respiration of Histoplasma capsulatum. Appl. Environ. Microbiol. 51: 65-73.
- LAVIE, S., and STOTZKY, G. 1986. Interactions between clay minerals and siderophores affect the respiration of Histoplasma capsulatum. Appl. Environ. Microbiol. 51: 74-79.
- LAWRENCE, J. R., and CALDWELL, D. E. 1987. Behavior of bacterial stream populations within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. 14: 15-27.
- LAWRENCE, J. R., DALAQUIS, P. J., KORBER, D. R., and CALDWELL D. E. 1987. Behavior of Pseudomonas fluorescens within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. 14: 1-14.
- LEICK, V. 1968. Ratios between contents of DNA, RNA and protein in different micro-organisms as a function of maximal growth rate. Nature 217: 1153-1155.
- LESSIE, T. G., and PHIBBS, P. V. 1984. Alternative pathways of carbohydrate utilisation in pseudomonads. Ann. Rev. Microbiol. 38: 359-87.
- LIEN, T., and KNUTSEN, G. 1976. Fluorometric determination of DNA in Chlamydomonas. Anal Biochem. 74: 560-566.
- LIPS, A., and JESSOP, N. E. 1979. Colloidal aspects of bacterial adhesion. In: Adhesion of Microorganisms to Surfaces, pp 5-28. Edited by D. C. Ellwood, J. Melling, and P. Rutter. Academic Press.
- LOEB, G. I. 1985. The properties of non-biological surfaces and their characterisation. In: Bacterial Adhesion to Surfaces, pp 111-129. Edited by D. C. Savage and M. Fletcher. Plenum Publishing Corp., New York.

- LYNCH, W. H., and FRANKLIN, M. 1978. Effect of temperature on the uptake of glucose, gluconate, and 2-ketogluconate by Pseudomonas fluorescens. Can. J. Microbiol. 24: 56-62.
- McCOY, W. F., and COSTERTON, J. W. 1982. Growth of sessile Sphaerotilus natans in a tubular recycle system. Appl. Environ. Microbiol. 43: 1490-1497.
- McCOY, W. F., and OLSEN, B. F. 1985. Fluorimetric determination of DNA concentration in municipal drinking water. Appl Environ. Microbiol. 49: 811-817.
- McELDOWNEY, S. 1984. Attachment of freshwater bacteria to solid surfaces. PhD Thesis, University of Warwick.
- McELDOWNEY, S., and FLETCHER, M. 1986a. Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces. J. Gen. Microbiol. 132: 513-523.
- McELDOWNEY, S., and FLETCHER, M. 1986b. Variability of the influence of physicochemical factors affecting bacterial adhesion to polystyrene substrata. Appl. Environ. Microbiol. 52: 460-465.
- MACKENZIE, A. M. R., and RIVIERA-CALDERON, R. L. 1985. Agar overlay method to measure adherence of Staphylococcus epidermidis to four plastic surfaces. Appl. Environ. Microbiol. 50: 1322-1324.
- MAEDA, M., and TAGA, N. 1979. Chromogenic assay method of lipopolysaccharide (LPS) for evaluating bacterial standing crop in seawater. J. Appl. Bacteriol. 47: 175-182.
- MARGREY, S. L., BURTON, D. T., RICHARDSON, L. B. and LIDEN, L. H. 1979. Artificial substrate system for biofouling studies. J. Environ. Eng. Div. 105: 161-164.

- MARSHALL, K. C. 1980. Bacterial adhesion in natural environments. In: Microbial Adhesion to Surfaces, pp 187-196. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, B. Vincent. Ellis Horwood, Chichester.
- MARSHALL, K. C. 1985. Mechanisms of bacterial adhesion at solid-water interfaces. In: Bacterial Adhesion to Surfaces, pp 133-161. Edited by D. C. Savage and M. Fletcher. Plenum Publishing Corp., New York.
- MARSHALL, K. C. 1986. Mechanisms of Bacterial Adhesion at Solid-Water Interfaces. In: Bacterial Adhesion to Surfaces, pp 133-161. Edited by D. C. Savage and M. Fletcher. Plenum Publishing Corp., New York.
- MARSHALL, K. C., STOUT, R., and MITCHELL, R. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. 68: 337-348.
- MARSHALL, K. C., and CRUIKSHANK, R. H. 1973. Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. Arch. Mikrobiol. 91: 29-40.
- MARSZALEK, D. S., GERCHAKOV, S. M., and UDEY, L. R. 1979. Influence of substrate composition on marine microfouling. Appl. Environ. Microbiol. 38: 987-995.
- MIDGLEY, M., and DAWES, E. A. 1973. The regulation of transport of glucose and methyl α -glucoside in Pseudomonas aeruginosa. Biochem. J. 132: 141-154.
- MOHANDAS, N., HOCHMUTH, R. M. and SPAETH, E. E. 1974. Adhesion of red cells to foreign surfaces in the presence of flow. J. Biomed. Mater. Res. 8: 119-136.
- MORIARTY, D. J. W., and POLLARD, P. C. 1982. Diel variation of bacterial productivity in seagrass (Zostera capricorni)

- beds measured by rate of thymidine incorporation into DNA. Mar. Biol. 72: 165-173.
- MUKKADA, A. J., LONG, G. L., and ROMANO, A. H. 1973. The uptake of 2-deoxy-D-glucose by Pseudomonas aeruginosa and its regulation. Biochem. J. 132: 155-162.
- NALBADIAN, J., FREEDMAN, M. L., TANZER, J. M., and LOVELACE, S. M. 1974. Ultrastructure of Streptococcus mutans with reference to agglutination, adhesion, and extracellular polysaccharide. Infect. Immunity 10: 1170-1179.
- NELSON, C. H., ROBINSON, J. A., and CHARACKLIS, W. G. 1985. Bacterial adsorption to smooth surfaces: rate, extent, and spatial pattern. Biotechnol. Bioeng. 27: 1662-1667.
- PAERL, H. W. 1985. Influence of attachment on microbial metabolism and growth in aquatic ecosystems. In: Bacterial Adhesion to Surfaces, pp 363-400. Edited by D. C. Savage and M. Fletcher. Plenum Publishing Corp., New York.
- PAUL, J. H. 1982. Use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria. Appl. Environ. Microbiol. 43: 939-944.
- PAUL, J. H. 1984. Effects of antimetabolites on the adhesion of an estuarine Vibrio sp. to polystyrene. Appl. Environ. Microbiol. 48: 924-929.
- PAUL, J. H., and MYERS, B. 1982. Fluorometric determination of DNA in aquatic microorganisms by use of Hoechst 33258. Appl. Environ. Microbiol. 43: 1393-1399.
- PAUL, J. H., and LOEB, G. I. 1983. Improved microfouling assay employing a DNA-specific fluorochrome and polystyrene as substratum. Appl. Environ. Microbiol. 46: 338-343.
- PAUL, J. H., and JEFFREY, W. H. 1985. Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic

- surfaces in Vibrio proteolytica. Appl. Environ. Microbiol. 50: 431-437.
- PEARCE, W. A., and BUCHANAN, T. M. 1980. Structure and cell membrane-binding properties of bacterial fimbriae. In: Bacterial Adherence, pp 289-344. Edited by E. H. Beachey. Chapman and Hall.
- PEDERSEN, K. 1982a. Method for studying microbial biofilms in flowing-water systems. Appl. Environ. Microbiol. 43: 6-13.
- PEDERSEN, K. 1982b. Factors regulating microbial biofilm development in a system with slowly flowing seawater. Appl. Environ. Microbiol. 44: 1196-1204.
- PERRY, R. H. and CHILTON, C. H. EDS. 1973. Chemical Engineers' Handbook 1973. 5th Ed. McGraw-Hill Kogakusha, Ltd.
- PETHICA, B. A. 1980. Microbial and cell adhesion. In: Microbial Adhesion to Surfaces, pp 19-45. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent. Ellis Horwood, Chichester.
- PHIBBS JR., P. V., and EAGON, R. G. 1970. Transport and phosphorylation of glucose, fructose, and mannitol by Pseudomonas aeruginosa. Arch. Biochem. Biophys. 138: 470-482.
- PICOLOGLOU, B. F., ZELVER, N., and CHARACKLIS, W. G. 1980. Biofilm growth and hydraulic performance. J. Hydraulic Div., Proc. Am. Soc. Civ. Eng. 106: 733-746.
- POWELL, M. S., and SLATER, N. K. H. 1982. Removal rates of bacterial cells from glass surfaces by fluid shear. Biotechnol. Bioeng. 24: 2527-2537.
- POWELL, M. S., and SLATER, N. K. H. 1983. The deposition of bacterial cells from laminar flows onto solid surfaces. Biotechnol. Bioeng. 25: 891-900.

- PRINGLE, J. H. 1983. PhD Thesis, University of Warwick.
- PRINGLE, J. H., and FLETCHER, M. 1983a. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. *Appl. Environ. Microbiol.* 45: 811-817.
- PRINGLE, J. H., FLETCHER, M., and ELLWOOD, D. C. 1983b. Selection of attachment mutants during the continuous culture of Pseudomonas fluorescens and relationship between attachment ability and surface composition. *J. Gen. Microbiol.* 129: 2557-2569.
- PRINGLE, J. H., and FLETCHER, M. 1986. Influence of substratum hydration and adsorbed macromolecules on bacterial attachment to surfaces. *Appl. Environ. Microbiol.* 51: 1321-1325.
- RHODES, M. E. 1959. The characterization of Pseudomonas fluorescens. *J. Gen. Microbiol.* 21: 221-263.
- RITTMANN, B. E. 1982. The effect of shear stress on biofilm loss rate. *Biotechnol. Bioeng.* 24: 501-508.
- RITTMANN, B. E., and McCARTY, P. L. 1980a. Model of steady-state-biofilm kinetics. *Biotechnol. Bioeng.* 22: 2343-2357.
- RITTMANN, B. E., and McCARTY, P. L. 1980b. Evaluation of steady-state biofilm kinetics. *Biotechnol. Bioeng.* 22: 2359-2373.
- RITTMANN, B. E., CRAWFORD, L., TUCK, C. K., and NAMKUNG, E. 1986. In situ determination of kinetic parameters for biofilms: isolation and characterization of oligotrophic biofilms. *Biotechnol. Bioeng.* 28: 1753-1760.
- ROBINS, R. J., HALL, D. O., SHI, D.-J., TURNER, R. J., and RHODES, M. J. C. 1986. Mucilage acts to adhere cyanobacteria and cultured plant cells to biological and inert surfaces. *FEMS Microbiol. Letts.* 34: 155-160.

- ROGERS, H. J. 1979. Adhesion of microorganisms to surfaces: some general considerations of the role of the envelope. In: Adhesion of Microorganisms to Surfaces, pp 29-56. Edited by D. C. Ellwood, J. Melling, P. Rutter. Academic Press.
- ROTH, I. L. 1977. Physical structure of surface carbohydrates. In: Surface Carbohydrates of the Prokaryotic Cell, pp 5-26. Edited by I. W. Sutherland. Academic Press, London.
- RUTTER, P. R., and VINCENT, B. 1980. The adhesion of microorganisms to surfaces: physico-chemical aspects. In: Microbial Adhesion to Surfaces, pp 79-92. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent. Ellis Horwood, Chichester.
- SANDERS, R. W., and PORTER, K. G. 1986. Use of metabolic inhibitors to estimate protozooplankton grazing and bacterial production in a monomictic eutrophic lake with an anaerobic hypolimnion. Appl. Environ. Microbiol. 52: 101-107.
- SAVAGE, D. C. 1984. Activities of microorganisms attached to living surfaces. In: Microbial Adhesion and Aggregation, pp 233-249. Edited by K. C. Marshall. Dahlem Konferenzen 1984. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.
- SHIEH, W. K. 1982. Mass transfer in a rotating biological contactor. Water Res. 16: 1071-1074.
- SIEGRIST, H., and GUJER, W. 1985. Mass transfer mechanisms in a heterotrophic biofilm. Water Res. 19: 1369-1378.

- SILVESTER, N. R., and SLEIGH, M. A. 1985. The forces on microorganisms at surfaces in flowing water. *Freshwater Biology* 15: 433-448.
- SIMON, M. 1985. Specific uptake rates of amino acids by attached and free-living bacteria in a mesotrophic lake. *Appl. Environ. Microbiol.* 49: 1254-1259.
- SINGLETON, P., and SAINSBURY, D. 1981. Introduction to bacteria. John Wiley and Sons Ltd.
- SMALL, D. A., MOORE, N. F., and ENTWISTLE, P. F. 1986. Hydrophobic interactions involved in attachment of a baculovirus to hydrophobic surfaces. *Appl. Environ. Microbiol.* 52: 220-223.
- STANLEY, P. M. 1983. Factors affecting the irreversible attachment of Pseudomonas aeruginosa to stainless steel. *Can. J. Microbiol.* 29: 1493-1499.
- SUIDAN, M. T., RITTMANN, B. E., and TRAEGER, U. K. 1987. Criteria establishing biofilm kinetic types. *Water Res.* 4: 491-498.
- SUTHERLAND, I. W. 1980. Polysaccharides in the adhesion of marine and freshwater bacteria. In: *Microbial Adhesion to Surfaces*, pp 329-328. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent. Ellis Horwood, Chichester.
- SUTHERLAND, I. W. 1982. Microbial exopolysaccharides - their role in microbial adhesion in aqueous systems. *CRC Critical Reviews in Microbiology* 10: 173-201.
- TADROS, F. 1980. Particle-surface adhesion. In: *Microbial Adhesion to Surfaces*, pp 93-116. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent. Ellis Horwood, Chichester.

- TOBIN, R. S., RYAN, J. F., and AFGHAN, B. L. 1978. An improved method for the determination of adenosine triphosphate in environmental samples. *Water Res.* 12: 783-792.
- TOBIN, R. S., and ANTHONY, D. H. J. 1978. Tritiated thymidine incorporation as a measure of microbial activity in lake sediments. *Limnol Oceanogr.* 23: 161-165.
- TUOVILA, B. J., and LAROCK, P. A. 1985. Effect of species difference and growth rate in the use of INT as an indicator of bacterial respiration. *J. Microbiol. Methods* 4: 185-188.
- TRULEAR, M. G., and CHARACKLIS, W. G. 1982. Dynamics of biofilm processes. *Journal W. P. C. F.* 54: 1288-1301.
- VAN DER MEI, H. C., WEERKAMP, A. H., and BUSSCHER, H. J. 1987. Physico-chemical surface characteristics and adhesive properties of Streptococcus salivarius strains with defined cell surface structures. *FEMS Microbiol. Letts.* 40: 15-19.
- VAN LOOSDRECHT, C. M., LYKLEMA, J., NORDE, W., SCHRAA, G., and ZEHNDER, A. J. B. 1987. The role of bacterial cell wall hydrophobicity in adhesion. *Appl. Environ. Microbiol.* 53: 1893-1897.
- VAN PELT, A. W. J., WEERKAMP, A. H., UYEN, M. H. W. J. C., BUSSCHER, H. J., DE JONG, H. P. and ARENDS, J. 1985. Adhesion of Streptococcus sanguis CH3 to polymers with different surface free energies. *Appl. Environ. Microbiol.* 49: 1270-1275.
- VERWEY, E.J.W., and OVERBEEK, J. T. G., 1948. *Theory of the stability of lyophobic colloids.* Elsevier, Amsterdam.

- VESPER, S. J. 1987. Production of pili (fimbriae) by Pseudomonas fluorescens and correlation with attachment to corn roots. Appl. Environ. Microbiol. 53: 1397-1405.
- VICENTE, M., de PEDRO, M. A., de TORRONTGUI, J., and CANOVAS, J. L. 1975. The uptake of glucose and gluconate by Pseudomonas putida. Mol. Cell. Biochem. 7: 59-64.
- WANNER, O., and GUJER, W. 1985. A multispecies biofilm model. Biotechnol. Bioeng. 28: 314-328.
- WARD, J. B., and BERKELEY, R. C. W. 1980. The microbial cell surface and adhesion to surfaces. In: Microbial Adhesion to Surfaces, pp 47-66. Edited by R. C. W. Berkeley, J. M. Melling, P. R. Rutter, and B. Vincent. Ellis Horwood, Chichester.
- WICHLACZ, P. L., and UNZ, R. F. 1985. Growth kinetics of attached iron-oxidizing bacteria. Appl. Environ. Microbiol. 50: 460-467.
- WICKEN, A. J. 1985. Bacterial cell walls and surfaces. In: Bacterial adhesion to surfaces, pp 45-71. Edited by D. C. Savage and M. Fletcher. Plenum Publishing Corp., New York.
- WRANGSTADH, M., CONWAY, P. L., and KJELLEBERG, S. 1986. The production and release of an extracellular polysaccharide during starvation of a marine Pseudomonas sp. and the effect thereof on adhesion. Arch. Microbiol. 145: 220-227.
- ZAIDI, B. R., BARD, R. F., and TOSTESON, T. R. 1984. Microbial specificity of metallic surfaces exposed to ambient seawater. Appl. Environ. Microbiol. 48: 519-524.
- ZOBELL, C. E. 1943. The effect of solid surfaces upon bacterial activity. J. Bacteriology 46: 39-56.

CHAPTER EIGHT

APPENDIX

1. Results of uptake experiments

Table 8.1 shows the results of all the experiments on uptake of attached biofilms which were exposed to various flow rates in the flow rig. The method for calculating glucose uptake per cell is detailed in Chapter 2 (page 71). For any given experiment, the uptake for tubes in the same 'leg' of the rig is shown as an average (unless the tubes were removed at different times). Standard deviations are shown so that the degree of variability between results for individual tubes can be seen.

Summary of all results from experiments using the flow rig

TABLE 8.1

Flow rate l/min	Glucose g/l in rig	Time in rig	Glucose uptake g/cell	Standard deviation	N	Date
0.2	0.02	30m	1.41×10^{-15}	6.03×10^{-16}	3	12/2/87
0.2	0.2	30m	7.69×10^{-15}	6.70×10^{-16}	2	25/3/87
0.2	0.2	1h	1.70×10^{-14}	-	1	23/9/86
0.25	0.5	30m	4.20×10^{-14}	4.61×10^{-16}	3	23/6/87
0.25	0.5	30m	2.22×10^{-14}	2.05×10^{-15}	3	25/6/87
0.2	2.0	30m	6.95×10^{-14}	-	1	11/9/86
0.2	2.0	30m	5.49×10^{-14}	-	1	18/9/86
0.2	2.0	30m	2.50×10^{-13}	3.49×10^{-14}	3	15/4/87
0.2	2.0	30m	1.10×10^{-13}	7.50×10^{-15}	3	30/4/87
0.2	2.0	30m	8.17×10^{-14}	5.23×10^{-15}	3	30/4/87
0.2	2.0	30m	2.64×10^{-13}	2.26×10^{-15}	3	7/5/87
0.2	2.0	30m	1.09×10^{-13}	1.06×10^{-14}	3	7/5/87
0.2	2.0	30m	2.63×10^{-13}	2.50×10^{-15}	3	28/5/87
0.2	2.0	1h	2.69×10^{-13}	-	1	18/9/86
0.2	2.0	1h	1.21×10^{-13}	-	1	7/10/86
0.2	2.0	1h	4.17×10^{-13}	-	1	9/10/86
0.5	0.2 ¹	1h	2.97×10^{-14}	1.22×10^{-14}	2	8/1/86
0.5	0.5 ²	1h	1.91×10^{-13}	-	1	31/10/85
0.5	0.5 ²	1h	1.81×10^{-13}	-	1	8/11/85
0.5	2.0	30m	5.49×10^{-14}	-	1	18/9/86
0.5	2.0	1h	2.23×10^{-13}	1.58×10^{-14}	2	16/1/85

¹ Cells grown, attached and used in rig at the concentration given, rather than being grown and attached in medium containing 2 g/l glucose.

² Cells grown in medium containing 2 g/l glucose, but during the attachment stage glucose concentration was the same as that used in the flow rig.

Flow rate l/min	Glucose g/l in rig	Time in rig	Glucose uptake g/cell	Standard deviation	N	Date.
0.5	20.0	30m	1.30×10^{-12}	-	1	4/9/86
0.7	2.0	30m	2.91×10^{-13}	4.73×10^{-14}	3	28/5/87
0.75	0.5	30m	3.45×10^{-14}	3.85×10^{-15}	3	23/6/87
0.75	0.5	30m	4.43×10^{-14}	1.62×10^{-14}	3	25/6/87
0.75	2.0	30m	1.38×10^{-13}	1.46×10^{-14}	3	2/7/87
1.0	0.02	30m	2.43×10^{-15}	1.70×10^{-16}	2	12/2/87
1.0	0.2	30m	1.11×10^{-14}	1.17×10^{-15}	3	25/3/87
1.0	0.2 ¹	1h	2.08×10^{-14}	1.39×10^{-15}	2	3/12/85
1.0	0.2	1h	3.55×10^{-14}	-	1	23/9/86
1.0	0.5	1h	1.86×10^{-14}	2.64×10^{-16}	2	21/11/85
1.0	2.0	30m	2.17×10^{-13}	-	1	11/9/86
1.0	2.0	30m	3.66×10^{-13}	-	1	18/9/86
1.0	2.0	30m	1.04×10^{-13}	-	1	9/10/86
1.0	2.0	30m	1.96×10^{-13}	3.84×10^{-14}	3	15/4/87
1.0	2.0	30m	4.04×10^{-13}	2.13×10^{-13}	3	15/4/87
1.0	2.0	30m	7.88×10^{-14}	5.24×10^{-15}	3	30/4/87
1.0	2.0	30m	8.63×10^{-14}	1.16×10^{-14}	3	30/4/87
1.0	2.0	30m	2.17×10^{-13}	3.48×10^{-14}	3	7/5/87
1.0	2.0	30m	3.38×10^{-13}	3.86×10^{-13}	3	7/5/87
1.0	2.0	30m	2.70×10^{-13}	3.50×10^{-14}	3	28/5/87
1.0	2.0	30m	2.77×10^{-13}	4.69×10^{-14}	3	28/5/87
1.0	2.0	30m	8.62×10^{-14}	1.82×10^{-14}	3	16/6/87
1.0	2.0	1h	2.51×10^{-13}	-	1	18/9/85
1.0	2.0	1h	1.67×10^{-13}	-	1	7/10/86
1.0	2.0	1h	1.24×10^{-13}	-	1	9/10/86
1.0	4.0 ¹	1h	5.01×10^{-15}	-	1	16/11/85

¹ Cells grown, attached and used in rig at the concentration given, rather than being grown and attached in medium containing 2 g/l glucose.

Flow rate l/min	Glucose g/l in rig	Time in rig	Glucose uptake g/cell	Standard deviation	N	Date
1.0	20.0 ²	1h	3.31×10^{-12}	1.44×10^{-12}	1	28/11/85
1.0	20.0	1h	1.47×10^{-12}	-	1	16/9/86
1.5	0.5	30m	4.53×10^{-14}	1.61×10^{-14}	3	23/6/87
1.5	0.5	30m	1.88×10^{-14}	1.61×10^{-14}	3	25/6/87
1.5	2.0	30m	1.61×10^{-13}	2.46×10^{-14}	3	2/7/87
2.0	0.02	30m	7.89×10^{-16}	8.63×10^{-16}	2	26/2/87
2.0	0.02	30m	8.63×10^{-16}	2.94×10^{-16}	2	26/2/87
2.0	0.02	1h	5.67×10^{-15}	5.68×10^{-16}	2	26/2/87
2.0	0.02	1h	4.59×10^{-15}	1.10×10^{-15}	2	26/2/87
2.0	0.02	30m	3.03×10^{-16}	1.22×10^{-16}	2	4/3/87
2.0	0.02	30m	3.82×10^{-16}	8.14×10^{-17}	2	4/3/87
2.0	0.02	1h	6.11×10^{-16}	3.03×10^{-17}	2	4/3/87
2.0	0.02	1h	8.92×10^{-16}	1.58×10^{-16}	2	4/3/87
2.0	0.2 ²	22m	3.56×10^{-15}	-	1	25/2/86
2.0	0.2 ²	22m	4.94×10^{-15}	-	1	19/2/86
2.0	0.2	36m	1.58×10^{-14}	-	1	11/3/87
2.0	0.2	36m	3.60×10^{-14}	5.58×10^{-15}	3	11/3/87
2.0	0.2	36m	2.65×10^{-14}	1.50×10^{-14}	3	11/3/87
2.0	0.2	45m	2.39×10^{-14}	8.39×10^{-15}	2	11/3/87
2.0	0.2	45m	1.96×10^{-14}	7.19×10^{-15}	3	11/3/87
2.0	0.2	45m	2.59×10^{-14}	4.08×10^{-14}	2	11/3/87
2.0	0.2 ²	50m	1.89×10^{-14}	-	1	19/2/87
2.0	0.2 ²	1h	1.61×10^{-14}	1.06×10^{-15}	2	3/12/85
2.0	0.2 ²	1h	2.90×10^{-14}	6.37×10^{-15}	2	8/1/86
2.0	0.2 ²	1h	1.17×10^{-14}	-	1	25/2/86

² Cells grown in medium containing 2 g/l glucose, but during the attachment stage glucose concentration was the same as that used in the flow rig.

Flow rate l/min	Glucose g/l in rig	Time in rig	Glucose uptake g/cell	Standard deviation	N	Date
2.0	0.2 ²	1h 24m	3.16×10^{-14}	-	1	19/2/86
2.0	0.2 ²	1h 45m	2.26×10^{-14}	-	1	25/2/86
2.0	0.2 ²	1h 54m	4.94×10^{-14}	-	1	19/2/86
2.0	0.2 ²	2h 22m	7.99×10^{-14}	-	1	19/2/86
2.0	0.2 ²	2h 31m	3.58×10^{-14}	-	1	25/2/86
2.0	0.2 ²	2h 50m	8.36×10^{-14}	-	1	19/2/86
2.0	0.2 ²	3h 5m	4.55×10^{-14}	-	1	25/2/86
2.0	0.2 ²	3h 35m	6.72×10^{-14}	-	1	25/2/86
2.0	0.5 ²	10m	7.84×10^{-14}	-	1	16/10/85
2.0	0.5 ²	10m	5.19×10^{-14}	-	1	24/10/85
2.0	0.5 ²	20m	2.74×10^{-14}	-	1	16/10/85
2.0	0.5 ²	20m	8.96×10^{-14}	-	1	24/10/85
2.0	0.5 ²	40m	2.00×10^{-13}	-	1	16/10/85
2.0	0.5 ²	40m	1.42×10^{-13}	-	1	24/10/85
2.0	0.5 ²	1h	2.13×10^{-13}	-	1	10/10/85
2.0	0.5 ²	1h	2.15×10^{-13}	-	1	31/10/85
2.0	0.5 ²	1h	8.56×10^{-14}	-	1	5/11/85
2.0	0.5 ³	1h	6.51×10^{-14}	3.57×10^{-13}	2	21/11/85
2.0	0.5 ²	2h	5.59×10^{-13}	-	1	10/10/85
2.0	0.5 ²	3h	1.09×10^{-12}	-	1	10/10/85
2.0	2.0	13m	4.33×10^{-13}	-	1	27/1/86
2.0	2.0	22m	1.42×10^{-13}	-	1	29/1/86
2.0	2.0	22m	2.55×10^{-13}	-	1	4/2/86
2.0	2.0	22m	1.34×10^{-13}	-	1	4/2/86

² Cells grown in medium containing 2 g/l glucose, but during the attachment stage glucose concentration was the same as that used in the flow rig.

³ Cells grown in medium containing 2 g/l glucose, but during the attachment stage the medium contained no glucose.

Flow rate l/min	Glucose g/l in rig	Time in rig	Glucose uptake g/cell	Standard deviation	N	Date
2.0	2.0	22m	1.72×10^{-13}	-	1	14/2/86
2.0	2.0	23m	9.08×10^{-13}	-	1	27/1/86
2.0	2.0	30m	1.29×10^{-13}	3.31×10^{-14}	3	16/6/87
2.0	2.0	30m	1.42×10^{-13}	1.25×10^{-14}	3	2/7/87
2.0	2.0	40m	7.56×10^{-13}	-	1	27/1/86
2.0	2.0	40m	1.26×10^{-12}	-	1	27/1/86
2.0	2.0	40m	2.92×10^{-13}	-	1	4/2/86
2.0	2.0 ⁴	40m	2.15×10^{-13}	-	1	4/2/86
2.0	2.0	1h	2.27×10^{-13}	8.15×10^{-14}	2	16/1/86
2.0	2.0	1h	1.29×10^{-12}	-	1	27/1/86
2.0	2.0	1h	1.73×10^{-13}	-	1	29/1/86
2.0	2.0	1h	2.76×10^{-13}	-	1	4/2/86
2.0	2.0	1h	2.32×10^{-13}	-	1	4/2/86
2.0	2.0	1h	1.98×10^{-13}	-	1	14/2/86
2.0	2.0	1h 50m	1.80×10^{-13}	-	1	14/2/86
2.0	2.0	2h	2.54×10^{-13}	-	1	29/1/86
2.0	2.0	2h 40m	3.13×10^{-13}	-	1	14/2/86
2.0	2.0	2h 45m	2.70×10^{-13}	-	1	29/1/86
2.0	2.0	3h 30m	3.30×10^{-13}	-	1	14/2/86
2.0	2.0	3h 35m	4.92×10^{-13}	-	1	29/1/86
2.0	2.0	5h 40m	5.74×10^{-13}	-	1	14/2/86
2.0	2.0	6h	9.50×10^{-13}	-	1	29/1/86
2.0	4.0	1h	3.16×10^{-13}	-	1	16/11/85
2.0	20.0	1h	3.03×10^{-12}	1.78×10^{-12}	2	28/11/85
2.5	0.5	30m	3.52×10^{-14}	3.64×10^{-13}	3	23/6/87
2.5	0.5	30m	2.28×10^{-14}	4.12×10^{-13}	3	25/6/87

⁴ Cells grown in medium containing 2 g/l glucose, but during the attachment stage glucose concentration was 1 g/l.

Flow rate l/min	Glucose g/l in rig	Time in rig	Glucose uptake g/cell	Standard deviation	N	Date
2.5	2.0	30m	1.49×10^{-13}	1.26×10^{-14}	3	2/7/87
3.0	0.02	30m	2.38×10^{-13}	6.51×10^{-14}	2	12/2/87
3.0	0.2	30m	7.34×10^{-13}	-	1	23/9/86
3.0	0.2	30m	9.38×10^{-13}	9.89×10^{-14}	3	25/3/87
3.0	0.2 ¹	1h	1.81×10^{-14}	3.52×10^{-13}	2	3/12/85
3.0	0.2 ¹	1h	2.85×10^{-14}	5.19×10^{-14}	2	8/1/86
3.0	0.2	1h	3.38×10^{-14}	-	1	23/9/86
3.0	0.5 ²	1h	3.94×10^{-13}	-	1	31/10/85
3.0	0.5 ²	1h	1.27×10^{-13}	-	1	5/11/85
3.0	0.5 ³	1h	9.92×10^{-14}	8.56×10^{-13}	2	21/11/85
3.0	2.0	30m	1.43×10^{-13}	-	1	11/9/86
3.0	2.0	30m	1.20×10^{-13}	-	1	18/9/86
3.0	2.0	30m	9.17×10^{-14}	-	1	7/10/86
3.0	2.0	30m	1.23×10^{-13}	-	1	9/10/86
3.0	2.0	30m	1.64×10^{-13}	5.03×10^{-14}	3	15/4/87
3.0	2.0	30m	1.64×10^{-13}	1.14×10^{-14}	3	16/6/87
3.0	2.0	30m	1.57×10^{-13}	1.31×10^{-13}	3	2/7/87
3.0	2.0	1h	2.07×10^{-13}	-	1	16/1/85
3.0	2.0	1h	1.54×10^{-13}	-	1	18/9/86
3.0	2.0	1h	1.76×10^{-13}	-	1	7/10/86
3.0	2.0	1h	5.32×10^{-13}	-	1	9/10/86
3.0	4.0 ¹	1h	1.46×10^{-13}	-	1	16/11/85

¹ Cells grown, attached and used in rig at the concentration given, rather than being grown and attached in medium containing 2 g/l glucose.

² Cells grown in medium containing 2 g/l glucose, but during the attachment stage glucose concentration was the same as that used in the flow rig.

³ Cells grown in medium containing 2 g/l glucose, but during the attachment stage the medium contained no glucose.

Flow rate l/min	Glucose g/l in rig	Time in rig	Glucose uptake g/cell	Standard deviation	N	Date.
3.0	20.0	1h	2.59×10^{-13}	4.14×10^{-13}	2	28/11/85
3.5	0.5	30m	2.83×10^{-14}	6.91×10^{-15}	3	23/6/87
3.5	0.5	30m	2.94×10^{-14}	1.50×10^{-14}	3	25/6/87
4.0	2.0	30m	1.66×10^{-13}	2.51×10^{-14}	2	2/7/87
4.4	0.5	30m	2.79×10^{-14}	2.22×10^{-15}	3	23/6/87
4.4	0.5	30m	8.13×10^{-14}	4.15×10^{-14}	3	25/6/87
4.4	0.5 ²	1h	2.01×10^{-13}	-	1	8/11/85
4.4	0.5 ²	1h	7.02×10^{-13}	-	1	8/11/85
4.4	2.0	30m	6.44×10^{-13}	2.36×10^{-13}	3	15/4/87
4.4	2.0	30m	8.07×10^{-14}	1.76×10^{-14}	3	30/4/87
4.4	2.0	30m	5.92×10^{-14}	1.95×10^{-14}	3	30/4/87
4.4	2.0	30m	2.15×10^{-13}	8.74×10^{-14}	3	7/5/87
4.4	2.0	30m	2.76×10^{-13}	2.38×10^{-13}	3	7/5/87
4.4	2.0	30m	2.33×10^{-13}	5.12×10^{-14}	3	28/5/87
4.4	2.0	30m	3.45×10^{-13}	3.66×10^{-14}	3	28/5/87

² Cells grown in medium containing 2 g/l glucose, but during the attachment stage glucose concentration was the same as that used in the flow rig.

2. Data for Psuedomonas fluorescens

Fig 8.1 shows growth curves for the Pseudomonas fluorescens cells used in this study, at various glucose concentrations.

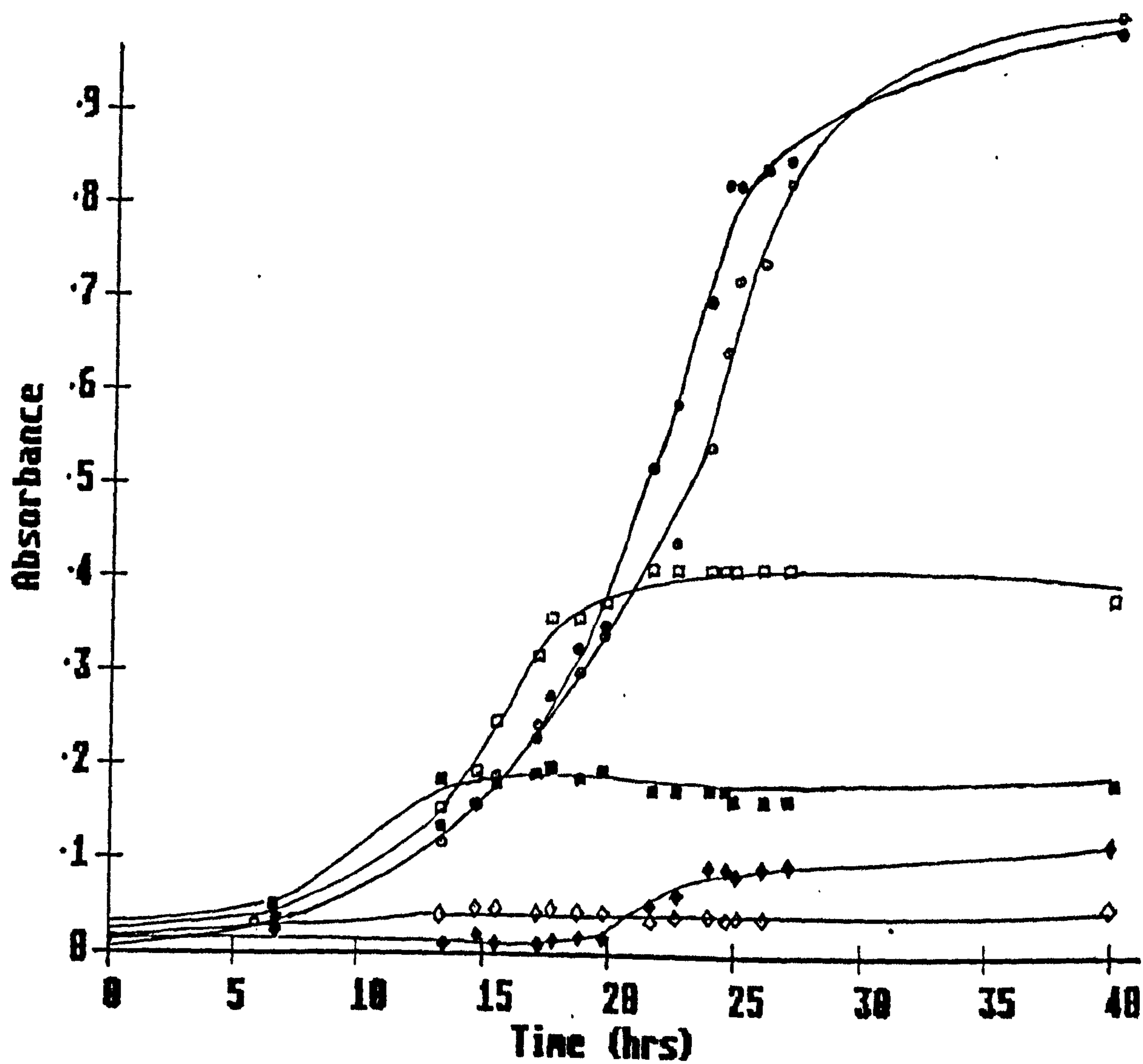
Figs 8.2 and 8.3 are calibration plots showing absorbance versus cell concentration, and cell numbers versus cell weight. The dry weight of a single cell was 2.09×10^{-13} g.

Glucose uptake by a growing culture is shown by Fig 8.4.

8.1 Growth curves for Pseudomonas fluorescens at different glucose concentrations.

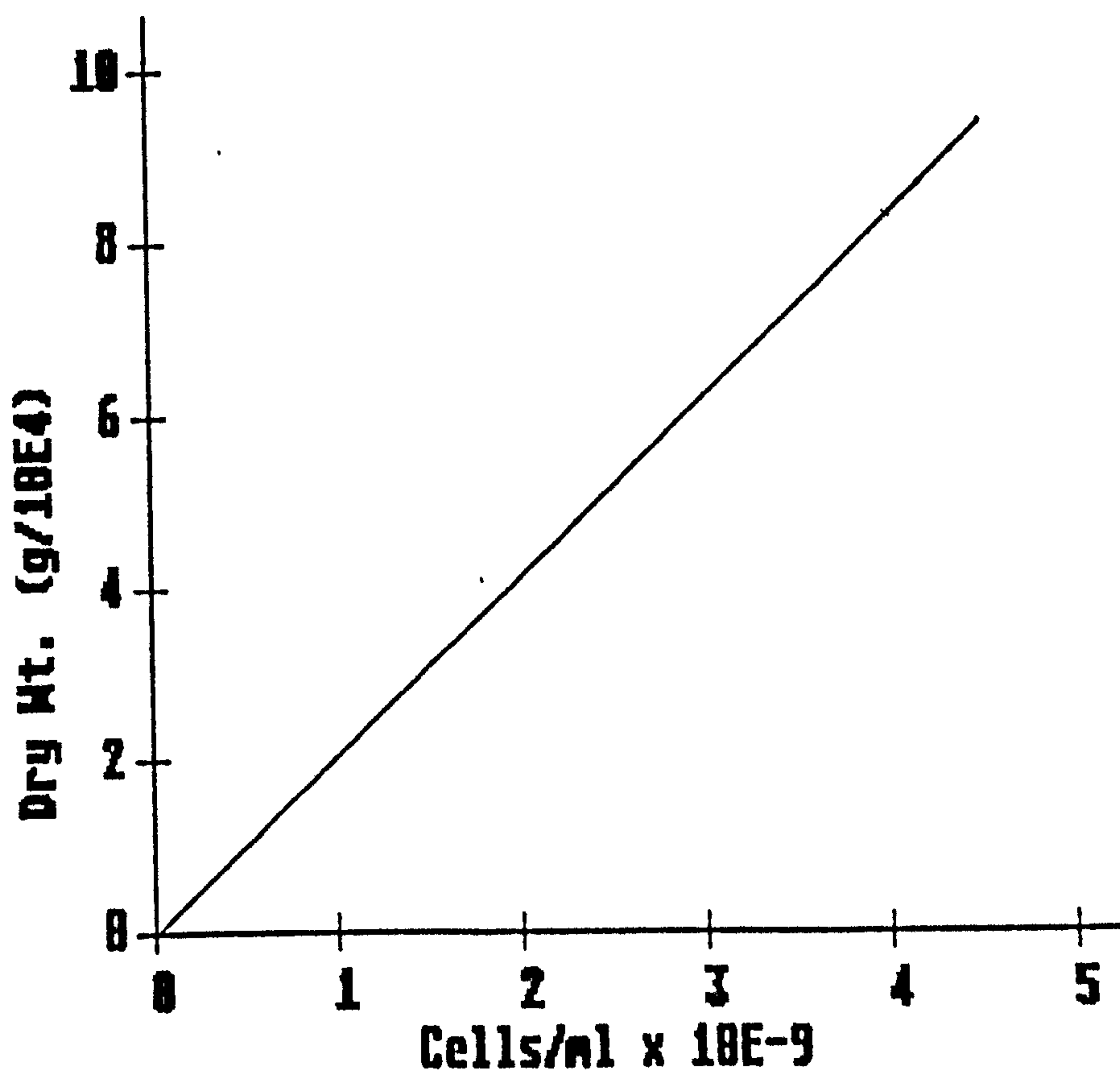
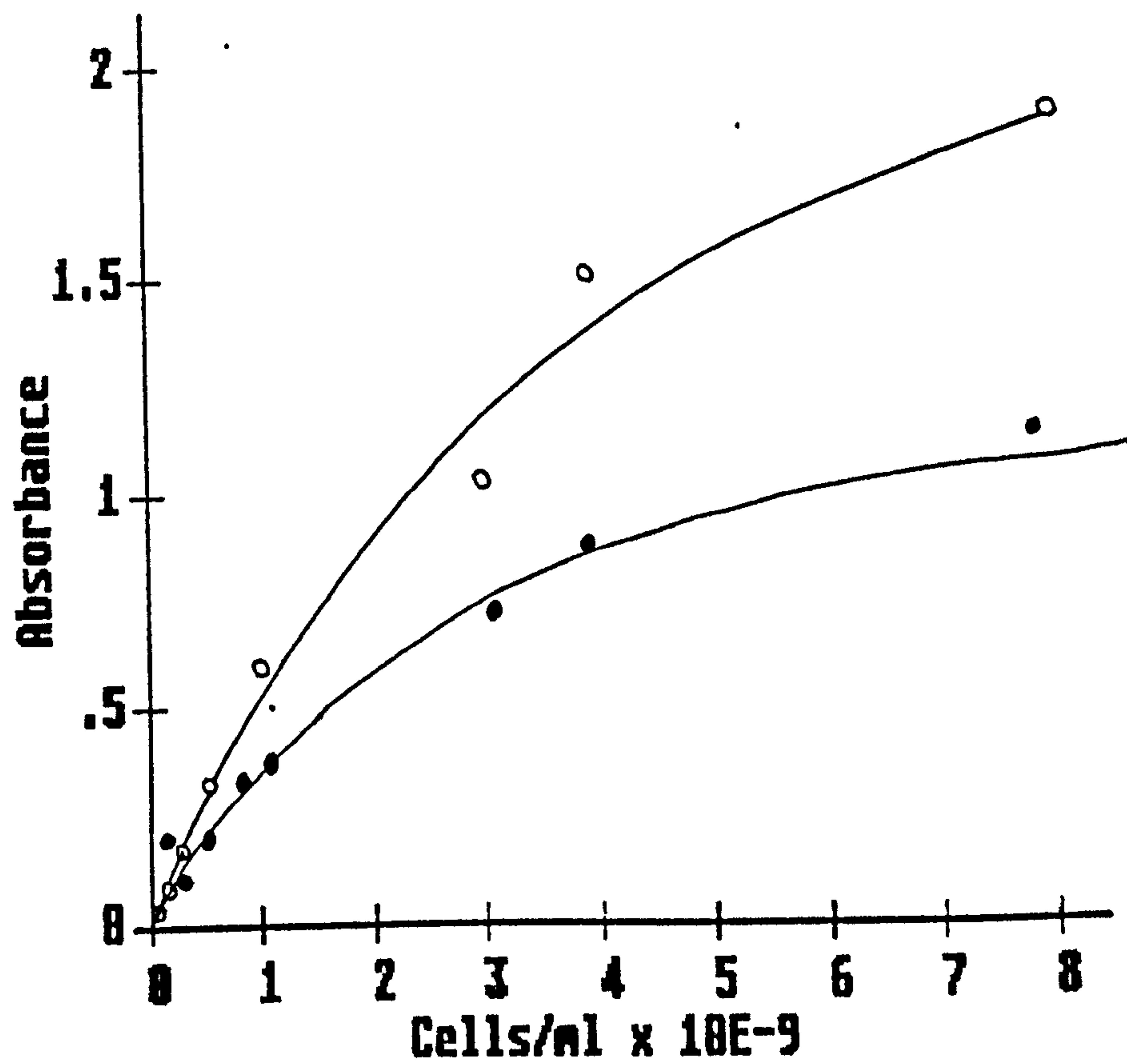
Key:

- 10.0 g/l glucose medium
- 2.0 g/l glucose medium
- ◻ 0.5 g/l glucose medium
- ◼ 0.2 g/l glucose medium
- ◆ 0.05 g/l glucose medium
- ◇ 0.02 g/l glucose medium



8.2 Calibration plot showing absorbance versus
concentration for Pseudomonas fluorescens.

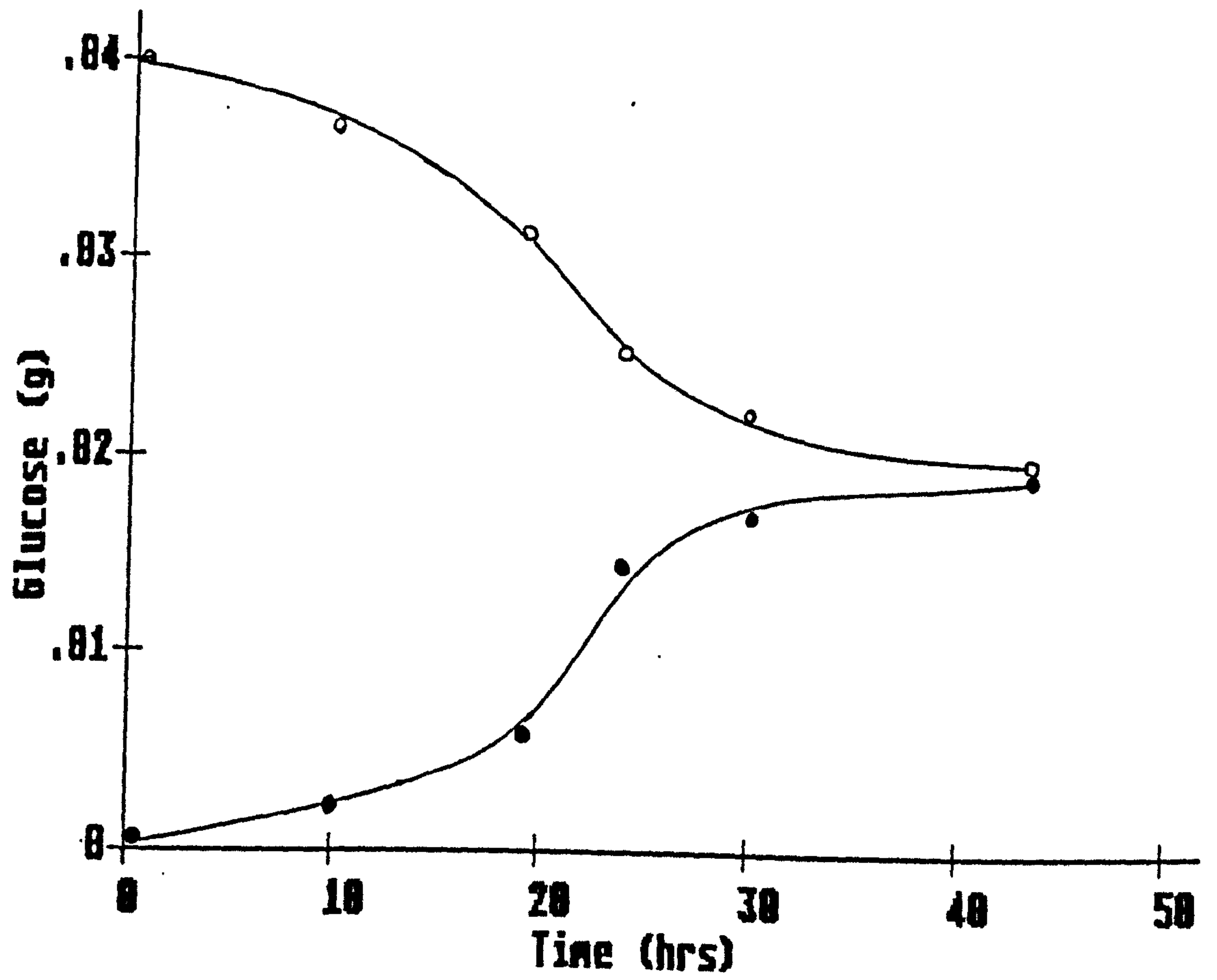
8.3 Plot showing cell weight versus cell numbers.



8.4 Glucose uptake by a growing culture.

Key:

- ☐ g/ml glucose in medium
- ☒ g glucose taken up by cells



3. Data on fluid flow characteristics in the test sections

3.1 Average velocity

The diameter of the test sections is 0.6 cm. The average velocity through the test sections is found by substituting into the equation:

$$u = Q/A$$

where u is the average velocity, Q is the flow rate, and A the cross-sectional area of the tubes ($=\pi r^2$). If the measured flow rate is 1.0 l/min, then:

$$\begin{aligned} Q &= 1.0/(60 \times 1000) \text{ m}^3/\text{s} \\ &= 1.67 \times 10^{-5} \text{ m}^3/\text{s}. \end{aligned}$$

$$\begin{aligned} \text{Therefore } u &= (1.67 \times 10^{-5})/(\pi \times (0.003)^2) \\ &= 0.58 \text{ m/s}. \end{aligned}$$

Fig 8.5 is a plot of flow rate versus average velocity through the test sections.

3.2 Reynolds number

The Reynolds number, Re , is calculated from the equation

$$Re = \rho u d / \mu$$

where fluid density, $\rho_{\text{H}_2\text{O}}$, is 999.0 kg/m³ and viscosity, $\mu_{\text{H}_2\text{O}}$, is 1.136×10^{-3} kg.m/s (Coulson and Richardson, 1954). Therefore, at a flow rate of 1.0 l/min, the Reynolds number is:

$$\begin{aligned} Re &= \frac{999.0 \times 0.58 \times 0.006}{1.136 \times 10^{-3}} \quad \frac{\text{kg.m}^{-3} \cdot \text{m.s}^{-1} \cdot \text{m}}{\text{kg.m}^{-1}\text{s}^{-1}} \\ &= 3108. \end{aligned}$$

Fig 8.6 is a plot of flow rate versus Reynolds number.

3.3 Peclet number

The Peclet number, N_{Pe} , is calculated from the equation

$$N_{Pe} = (v/D) \times Re$$

where ν is the kinematic viscosity (cm^2/s), and D is diffusivity of the substrate (Shieh, 1982). If the flow rate equals 1.0 l/min, then:

$$N_{pe} = \frac{(1.136 \times 10^{-3}/999.0) \times 3108}{6.0 \times 10^{-8}} \quad (\text{m}^2/\text{s})/(\text{m}^2/\text{s})$$

$$= 6.69 \times 10^4.$$

3.4 Calculation of shear stress at the pipe wall

Shear stress at a pipe wall, τ_w , can be calculated using the expression:

$$\tau_w = C_f \cdot \rho \cdot u^2 / 2$$

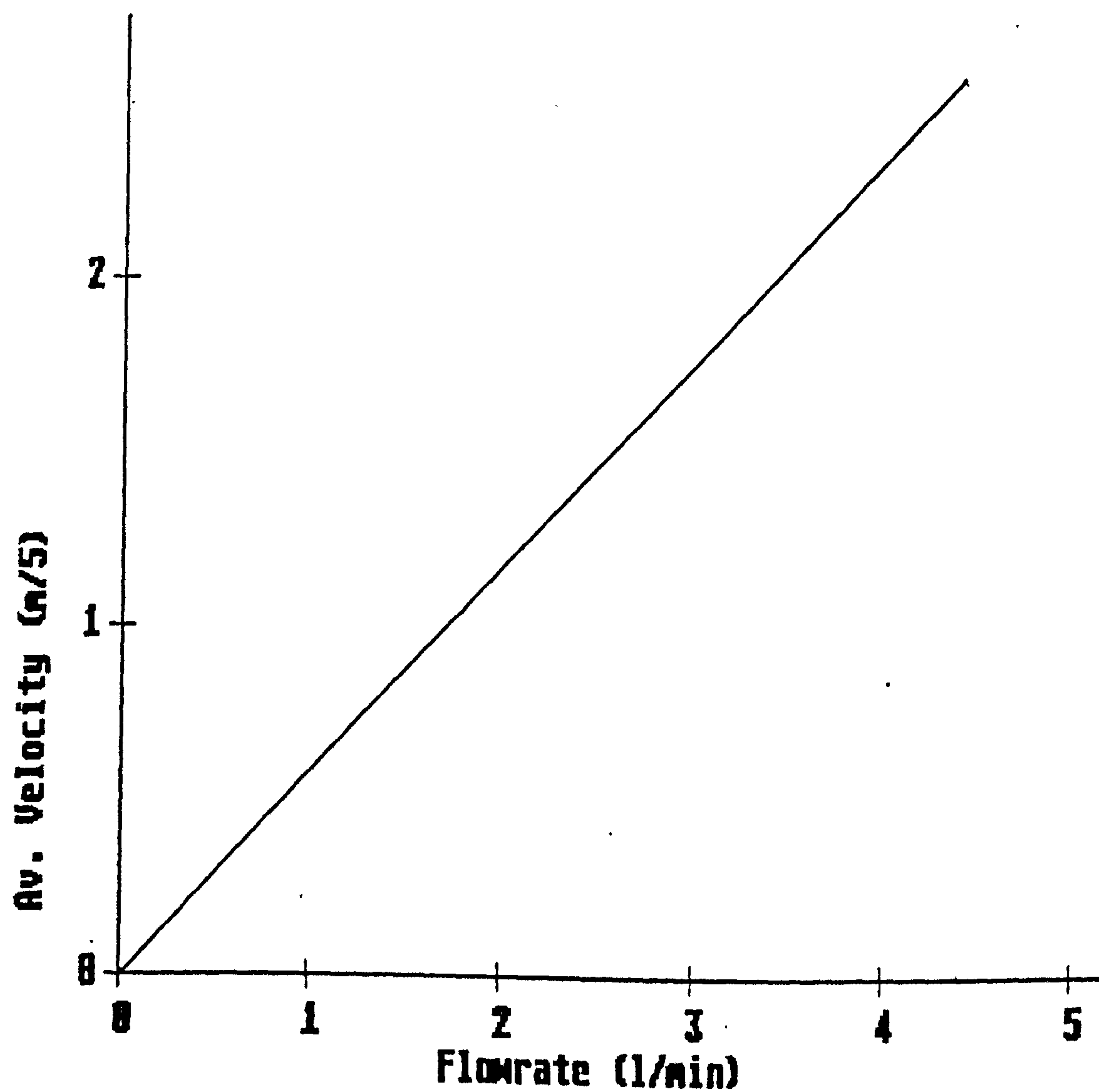
Values of $C_f/2$ are given in Chemical Engineering (Coulson and Richardson, 1954). for a flow rate of 1.0 l/min, the wall shear stress is:

$$\tau_w = (0.0054 \times 999.0 \times (0.58)^2) / 2 \quad \text{kg/m}^3 \cdot (\text{m/s})^2$$

$$= 1.84 \text{ kg} \cdot \text{m}^{-1} \cdot \text{s}^{-2}$$

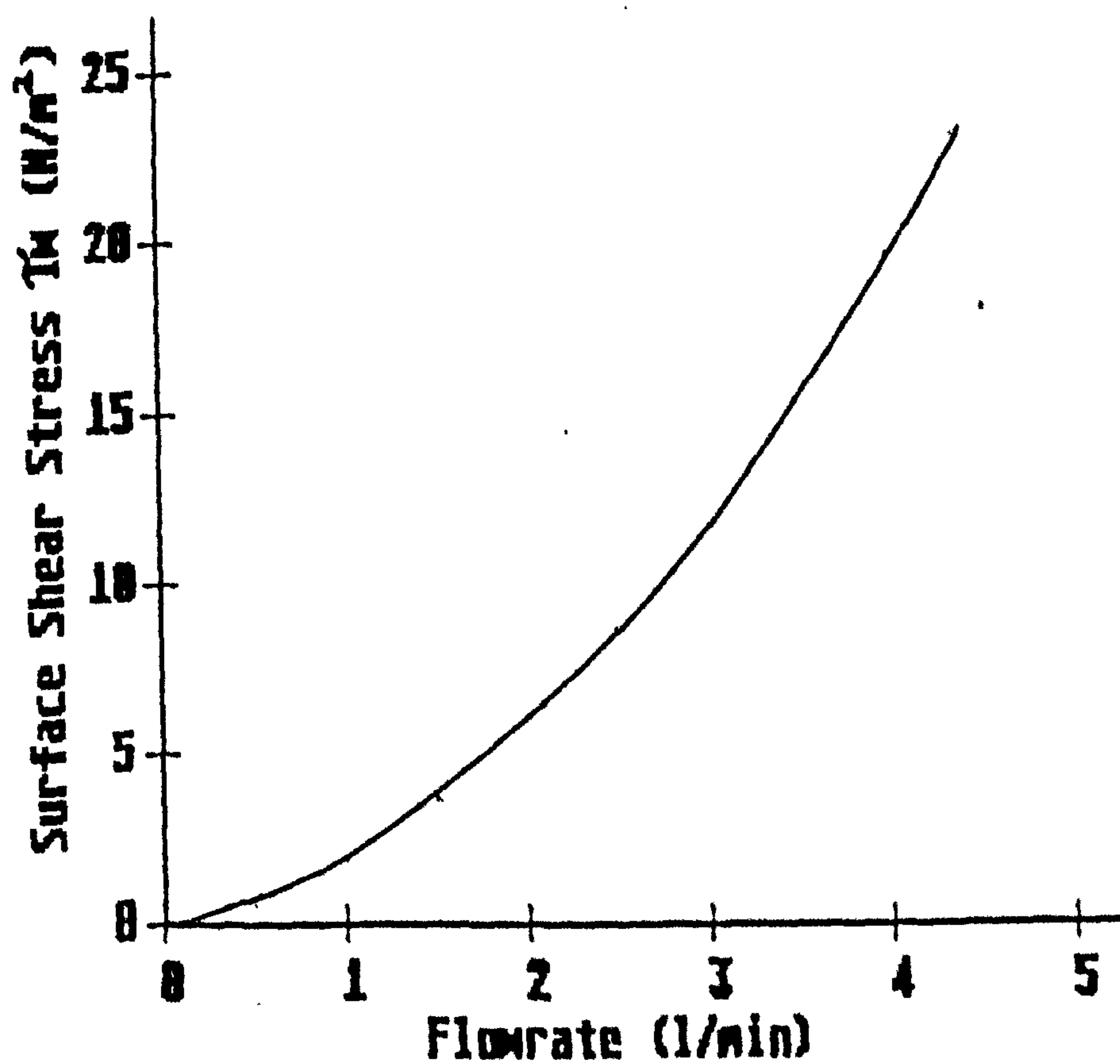
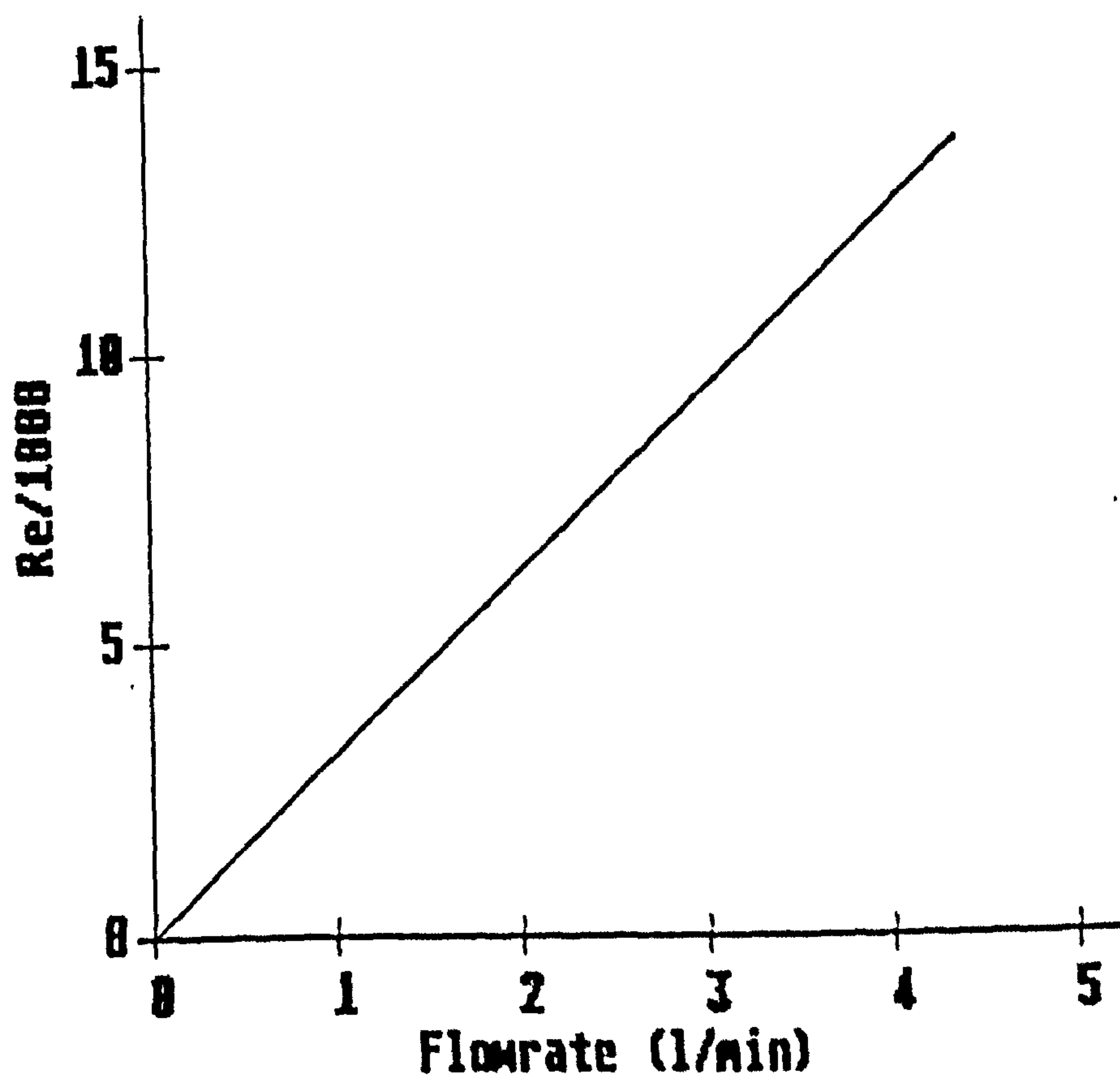
Fig 8.7 is a plot of flow rate versus surface shear stress.

8.5 Flow rate versus average velocity through test
 sections.



8.6 Flow rate versus Reynolds number.

8.7 Flow rate versus surface shear stress.



3.5 Velocity profiles

Velocity profiles can be calculated for the test sections if the flow is assumed to be fully developed, ie the velocity profile is the same at all points in the test sections (see discussion on page 129). Although the flow in the test sections is probably not fully developed, the changes in the velocity profile in the test sections are likely to be small, as the most rapid changes will occur just after the fluid is constricted into the 6 mm diameter tubing, before the test sections. It is therefore a reasonable approximation to regard the flow as being fully developed.

3.5.1 Laminar flows

For laminar flows, the velocity profile across the test sections can be calculated from the following expression, which is derived from the Navier Stokes equations:

$$u = ((P/L) \times (r^2 - r_o^2))/4$$

where r is the distance from the centre of the tube, r_o is the radius of the tube, and P/L is the pressure drop per unit length. The latter can be found using the Fanning equation (Perry and Chilton, 1973):

$$- P/L = 2.C_f \cdot \rho \cdot u_{av}^2 / d$$

If the flow rate through the test sections is 0.2 l/min, then u_{av} is 0.118 m/s (from Fig 8.5). Therefore:

$$\begin{aligned} - P/L &= 2 \times 0.026 \times 999.0 \times (0.118)^2 / 0.006 \\ &\quad (\text{kg} \cdot \text{m}^{-3} \cdot \text{m} \cdot \text{s}^2 / \text{m}) \\ &= 120.3 \text{ kg/m}^3 \cdot \text{s}^2 \end{aligned}$$

Therefore, at a distance of 1 m from the tube wall,

$$u = \frac{-120.3 \times ((0.003 - (1 \times 10^{-4}))^2 - (0.003)^2)}{4 \times (1.136 \times 10^{-3})}$$

$$(\text{kg.m}^{-3}.\text{s}^{-2}.\text{m}^2/\text{kg.m}^{-1}.\text{s}^{-1})$$

$$u = 1.59 \times 10^{-4} \text{ m/s}$$

Fig 4.9 (page 142) shows the velocity profile for distances up to 10 μm from the tube surface (about ten times the thickness of the attached cells).

3.5.2 Turbulent flows

For turbulent flows the velocity profile across the pipe can be calculated using Prandtl's seventh power law (Coulson and Richardson, 1954) or the universal velocity profile (Coulson and Richardson, 1954). The former is not accurate at the pipe wall, which is of the most significance to the attached cells. In addition u_{av} is assumed to be $0.82u_{\text{max}}$ (the velocity at the centre to the pipe) which is only true at very high Reynolds numbers. The universal velocity profile was therefore used. Separate relationships are used to predict the velocity in the laminar sublayer, close the the pipe wall, the buffer layer and the fully turbulent regions.

The velocity in the laminar sublayer at a distance y from the centre of the tube, u_y , is calculated using the relationship

$$u_y = u^+.u^*, \text{ where } u^+ = \rho u^* y / \mu = y^+;$$

$$u^* = \sqrt{(R/\rho)}; \quad y^+ = (y \sqrt{(R/\rho)}) / \mu, \text{ and } R = -R_w$$

This equation applies up to values of up to $y^+ = 5$. The shear stress at the pipe wall, R_w , is found from the Blasius

equation² (Coulson and Richardson, 1954).

Fig 4.9 (page 142) shows velocity profiles for distances up to 10 μ m from the tube surface. Fig 4.10 (page 143) shows the thickness of the laminar sublayer at various flow rates.